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On the Digestion and Absorption of Lipoperoxides

By

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Abstract

GLAVIND, J. and N. TRYDING. *On the digestion and absorption of lipoperoxides.* Acta physiol. scand. 1960. *49*. 97—102. — The purpose of the work was to study the fate of orally ingested peroxides. Lipoperoxides were given by mouth to rats with cannulated thoracic duct. No lipoperoxide could be found in the lymph. Pancreatic juice, bile, and lymph were shaken with lipoperoxides at suitable temperature and pH. At intervals free fatty acids, total lipoperoxide, and, after chromatographic separation on alumina, peroxide-containing esters and peroxide-containing free fatty acids were determined. Pancreatic juice splits lipoperoxides with the formation of peroxide-containing fatty acids, and generally also produces a decrease of total lipoperoxide. Bile and lymph alone have no effect on lipoperoxides. The results suggest that the essential site of the destruction of the lipoperoxides ingested with the food is the intestinal mucosa.

The normal diet regularly contains a larger or smaller quantity of lipoperoxides (lipids containing peroxide-groups). On the other hand, the lipids from different parts of the organism only contain negligible amounts of peroxides. This general observation is confirmed by experiments. DUBOULOZ, FONDARAI and LAGARDE (1949) fed large amounts of lipoperoxides to rats, but none could be recovered in the adipose tissue. We have made similar experiments with the same result. Such studies suggest the existence of a mechanism for the elimination

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of lipoperoxides consumed with the food. The purpose of the experiments reported in this paper was to identify the site of this mechanism which is even less understood than the mechanism by which lipoperoxide formation in tissues is prevented.

DUBOULOZ *et al.* (1949) studied the fate of orally ingested lipoperoxides in the digestive tract. They found a gradual disappearance in the course of some hours, roughly paralleling the course of fat absorption. We have made similar studies. Since they confirm DUBOULOZ' results they shall not be reported in detail.

After having passed through the intestinal wall the long chain fatty acids are transported *via* the thoracic duct into the blood-stream. For that reason animals in which the thoracic duct has been cannulated are well suited for following fat absorption. In the present study, the fate of orally ingested lipoperoxides in rats with cannulated thoracic duct is reported. Furthermore, we report some *in vitro* experiments on the influence of pancreatic juice, bile, bile acids, and lymph on lipoperoxides.

Material and Methods

The animals used were adult male white rats weighing about 250 g. The cannulation of the thoracic duct and the post-operative treatment were carried out according to BERGSTRÖM, BLOMSTRAND and BORGSTRÖM (1954). A mixture of bile and pancreatic juice was obtained by inserting a cannula into the lower end of the bile duct of the rat (cf. BORGSTRÖM 1952). Pure pancreatic juice was collected from the lower end of the bile duct, pure bile being simultaneously diverted from the upper end of the duct.

Preparation of lipoperoxides. Air was blown through olive oil and a sample of pure ethyl linoleate until the lipoperoxide content as followed by daily determinations had risen to about 1,000 μeq per g. For the preparation of peroxide-containing fatty acids 50 g of olive oil having about 1,000 μeq of peroxide per g were shaken for 24 hours at 37° C with a solution of 5 g of pancreatin (Novo) in 300 ml tris-buffer of pH 7.8. The lipids were extracted with petrol ether after adjusting pH to 3. The petrol ether solution was extracted twice with 92 % (v/v) methanol. An equal volume of water was added to the combined methanol phases, the lipids were extracted with ethyl ether, and the ether evaporated. An oil with a peroxide content of 1,650 μeq per g was obtained. When the peroxides bound to the free fatty acids were separated from those bound to glycerides by the method described below, it was found that the peroxide was about equally distributed between the two fractions.

Determination of lipoperoxides. The colorimetric thiocyanate method (GLAVIND and HARTMANN 1955) was used.

Separation of peroxide-containing free fatty acids from peroxide-containing esters. The two groups of substances were separated by chromatographic absorption, the former being more firmly absorbed. The method described in the following did not give a completely quantitative recovery but a good separation was obtained. A column of purified alumina (GLAVIND and HARTMANN 1955) was prepared, the lipid mixture dissolved in chloroform passed through, and the column washed with more solvent (pharmacopoea preparations of chloroform containing 0.6—1.0 % (v/v) ethanol were used). The peroxide-containing esters were found in the filtrate where the amount was determined after evaporation to a small volume at low temperature *in vacuo.* The peroxide-containing free fatty acids remaining on the column were eluted with chloroform containing 1 % (v/v) acetic acid.

After evaporation at low temperature to a small volume this fraction of the lipoperoxides could be determined by the usual method.

Experiments with pancreatic juice. The enzyme solution was adjusted to a suitable pH for pancreatic lipase (Borgström 1953) and shaken with the lipoperoxide in a flask at 37° C. Samples were taken at intervals. The pH of the samples was adjusted to 3 by the addition of 0.1 N HCl, and the lipid extracted with chloroform. After drying with anhydrous sodium sulphate the extracts were analyzed for dry-matter, free fatty acids (cf. Borgström 1952), total peroxide, peroxide in ester fraction, and peroxide in free fatty acids.

Experiments with bile, crystalline bile acids, and lymph were carried out in the same way as the experiments with pancreatic juice. Pure fistula bile and fistula lymph obtained from rats as described above were used. Furthermore, the following crystalline bile acids were tested: cholic acid, glycodesoxycholic acid, and taurocholic acid. These acids were kindly supplied by Dr. A. Norman.

Results

The absorption of lipoperoxides in rats with cannulated thoracic duct. Seven experiments were carried out. In two of them 0.5 ml ethyl linoleate containing about 600 μeq of peroxide was given. Four experiments were carried out in which 1 ml olive oil with a peroxide content of about 1,000 μeq was ingested, and finally one experiment was made using 1.1 ml of the above-mentioned product containing 1,650 μeq of peroxide per g, partly bound to free fatty acids.

The peroxide-containing oils were introduced into the stomach of the rats through a rubber tube. Lymph samples were collected separately through every whole hour during the first 12 hours period and in three of the experiments also during the next 12 hours after the administration. The samples were analyzed separately. The fat was extracted by grinding with chloroform and anhydrous sodium sulphate in a mortar. The sodium sulphate was filtered off, the chloroform evaporated, and peroxide determined as described above.

The results of all the experiments showed that none of the lymph samples contained lipoperoxides in detectable quantities.

In the faeces collected daily during four days from three of the rats and analyzed for peroxide content, less than 0.5 per cent of the ingested amount could be recovered.

The action of pancreatic juice, bile, bile acids, and lymph on lipoperoxides. The action of pancreatic juice on lipoperoxides was studied in two types of experiments, one with pancreatic juice alone, and the other with pancreatic juice and bile together. Table I and II show representative examples of each type of experiment.

As seen from Table I and II the total amount of peroxide in free fatty acids and esters put together was somewhat less than the total peroxide content found by direct determination without separation. This is in accordance with recovery experiments with mixtures of known quantities of the two kinds of lipoperoxides which always reveal losses, especially of peroxide-containing free fatty acids.

Table I. The action of pancreatic juice on lipoperoxides. Pancreatic juice was adjusted to pH 8 with tris-buffer and shaken with peroxide-containing olive oil at 37° C

Duration of experiment, hours	Peroxide, μeq/g total lipids, in			Free fatty acids, per cent of total lipids
	Total lipids	Free fatty acids	Esters	
0	630	35	600	10
1	615	60	430	34
2	430	110	270	49
3	260	60	160	51
4	245	70	100	61

Table II. The action of bile-pancreatic juice on lipoperoxides. The mixture of bile and pancreatic juice was adjusted to pH 5.2 with 0.1 N HCl and shaken with peroxide-containing olive oil at 37° C

Duration of experiment, hours	Peroxide, μeq/g total lipids, in			Free fatty acids, per cent of total lipids
	Total lipids	Free fatty acids	Esters	
0	430	18	410	4
0.5	233	15	213	7
1	230	21	185	10
2	215	35	155	18
3	210	45	125	26

Both tables show that incubation of lipoperoxides with pancreatic juice, alone or in combination with bile, resulted in a decrease in the total amount of peroxide groups. In other experiments sometimes a larger, sometimes a smaller decrease, sometimes no change in peroxide content was found. After 24 hours a significant quantity always remained. The decrease affected both the free fatty acid and the ester fractions although the rate of reduction may be different.

The proportion of peroxide groups bound to the free fatty acids in relation to those bound to free fatty acids and esters put together increased through the action of pancreatic juice. In the course of the experiments it increased in a manner roughly paralleling the liberation of free fatty acids as followed by titration with sodium hydroxide.

In the experiments carried out with pure bile without the contamination of pancreatic juice no effect on the peroxide content was found, neither on the total amount of peroxide nor on the partition between free fatty acids and esters. The same result was found with crystalline bile acids. Since all the experiments gave a negative result, no details shall be given.

Similarly no details shall be reported from the experiments with lymph. No effect of rat fistula lymph on peroxides was found.

Discussion

The results show quite clearly that orally ingested lipoperoxides cannot be recovered in the lymph. The pancreatic lipase hydrolyzes the peroxide-containing lipoperoxides so that fatty acids containing peroxide-groups are set free. Furthermore, through the action of the pancreatic juice a partial reduction of the peroxide groups takes place. Only very small amounts can be recovered from the faeces.

DUBOULOZ *et al.* (1949) incline to the theory that the metabolism of the lipoperoxides is the function of all cells of the organism. They have carried out several studies on the nature of the ubiquitous peroxide-destroying principle, especially the substances found in the liver. We have also studied this peroxide-destroying principle from liver, and, in general, we have been able to confirm DUBOULOZ' results. It should be taken into consideration, however, that the peroxide group is highly reactive to a great variety of substances, and evidence for the existence of a more specific system which metabolizes or detoxicates the lipoperoxides is lacking. From the results mentioned above it seems doubtful whether any lipoperoxide from a nutritional source ever reaches the liver or any other parenchymatous tissue beyond the intestinal mucosa. Thus, doubt is thrown upon the physiological role of DUBOULOZ' peroxide-destroying principle, at least so far as concerns the destruction of orally ingested peroxides or peroxides modified by the action of the pancreatic lipase.

Some destruction of lipoperoxides probably takes place in the digestive tube although, for the present, the extent of the peroxide destruction cannot be evaluated. However, through the action of the pancreatic lipase also peroxide-containing fatty acids are set free from the esters. Presumably the peroxides bound to the free fatty acids and esters are mainly absorbed and destroyed in the intestinal mucosa.

All studies so far reported are in accordance with the theory that although the lipoperoxides partially disappear in the digestive tract, the ultimate site of the "de-peroxydation" of the lipids is the intestinal mucosa.

DUBOULOZ, CHANCEL-GANDONNIÈRE and MARVILLE (1958) consider the presence of a peroxide-destroying enzyme in this tissue improbable because of their observation that intestine heated to 100° C destroyed as much peroxide as did fresh intestine. This result is further evidence against DUBOULOZ' view of his peroxide-destroying principle as the normal vehicle for the elimination of lipoperoxides from nutritional sources. On the other hand, the theory that the elimination of orally ingested lipoperoxides takes place in the intestinal mucosa is in conformity with the central role played by that tissue in the absorption and transformation of fats.

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The Effect of Oxytocic Substances upon the Rabbit Uterus in Situ

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Abstract

FUCHS, A.-R. and F. FUCHS. *The effect of oxytocic substances upon the rabbit uterus in situ.* Acta physiol. scand. 1960. *49*. 103—113. — On oestrogen-treated virginal rabbits the threshold dose of oxytocin varied between 0.005 and 0.05. No definite threshold could be established for methylergometrine. The difference between the lowest dose giving repeatable response and that giving maximal effect was very small. The higher dose had an adverse effect upon the whole animal. Dihydroergotamine did not show any oxytocic effect, nor did it influence oxytocin-induced contractions. On puerperal rabbits the sensitivity to oxytocin was still much increased 22 hours after delivery, but diminished during the following days. For methylergometrine the lowest dose with repeatable effect was the same as in the non-pregnant animal but the response was stronger. Dihydroergotamine had no effect upon the uterus. Isometric and isotonic recording gave the same results.

Natural ergot alkaloids and some of their semisynthetic derivatives have been extensively used as oxytocics in clinical obstetrics. However, their pharmacological action has not yet been completely clarified. Many observations indicate that differences between the action of these substances and that of the pituitary oxytocin may exist.

All species so far studied react in the same way to pituitary oxytocin and to comparable doses (REYNOLDS 1949), whereas great species variations are observed with regard to methylergometrine and other ergot alkaloids. In the human methylergometrine is clinically effective according to numerous authors, but only 13 out of 30 *in vitro* strips of human uteri at term were found to con-

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tract on this substance (Rothlin and Berde 1954). Furthermore, the minimal effective doses (2—4 mg/l bath fluid) were quite high as compared with the dose used in clinical practice (0.2 mg/patient i. v. or i. m.). In the rabbit, on the other hand, lower doses are effective *in vitro* (0.3 mg/l), while *in vivo* the effect is not clear (David and Kirchhof 1947). Votava and Podvalova (1957) found that with 0.1 mg/kg the onset of contractions was uncertain, and Rothlin and his coworkers used 0.4 mg/kg to obtain effect in the live rabbit. In the guinea pig, on strips of "responsive uteri", methylergometrine and ergometrine had equal effect in doses of 6.7 mg/l fluid, while neither strips of rat uteri nor of virgin dog uteri contracted on these drugs (David and Kirchhof 1947). In the puerperium Swanson and Hargreaves (1934) found the dog's uterus reactive to ergot alkaloids.

With regard to the hydrogenated ergot alkaloids similar species differences exist. Rothlin (1946) held that they inhibit uterine contractions, judging from rabbit experiments. Later Berde and Rothlin (1953) found a strong oxytocic effect in cats *in vivo*. Dihydroergotamine was thought to have no oxytocic effect on the human uterus, but in 1952 such an effect was observed by Altman *et al.* during labour and confirmed by Rothlin and Berde (1954) *in vitro* and by Embrey and Garrett (1955) *in vivo*. The hydrogenated ergot alkaloids also have a marked adrenaline-inhibiting effect upon the human uterus *in vitro* (Rothlin and Berde 1954), but *in vivo* Garrett (1955) was unable to demonstrate any such inhibition.

Until recently all studies of the pharmacological effect upon the uterus have been carried out by recording isotonic shortening. As pointed out by Csapo (1954) a quantitative evaluation of the activation of the myometrium can only be obtained by recording either isometric tension development or isotonic shortening with *optimal load*. Since the load used has not been stated in previous studies the results of different investigators are difficult to compare.

The aim of the present work has been to study the effect of some ergot alkaloids on the rabbit uterus *in vivo* under reproducible conditions, and to compare it with the effect of pituitary oxytocin.

Methods

Isometric tension development has been recorded *in situ* by the method described by Schofield (1954). A plexiglass chamber is placed in a midline incision in the lower abdomen of the supine rabbit, and a segment weighing around 2 g of one of the intact uterine horns is fixed within the chamber. Around the middle of the segment a loose loop of silk is tied with the free end passing through a funnel at the top of the chamber and pulling on an isometric myograph. This consists of a flat watch spring with a short and a long lever with a length ratio of 1: 30, giving a suitable curve on the kymograph with a negligible shortening of the uterine segment. The recordings were made with the uterine segment at resting length which was determined at the beginning of the experiment. The sensitivity of the recording was found to be one g at 50 g tension and two g at 100 g tension.

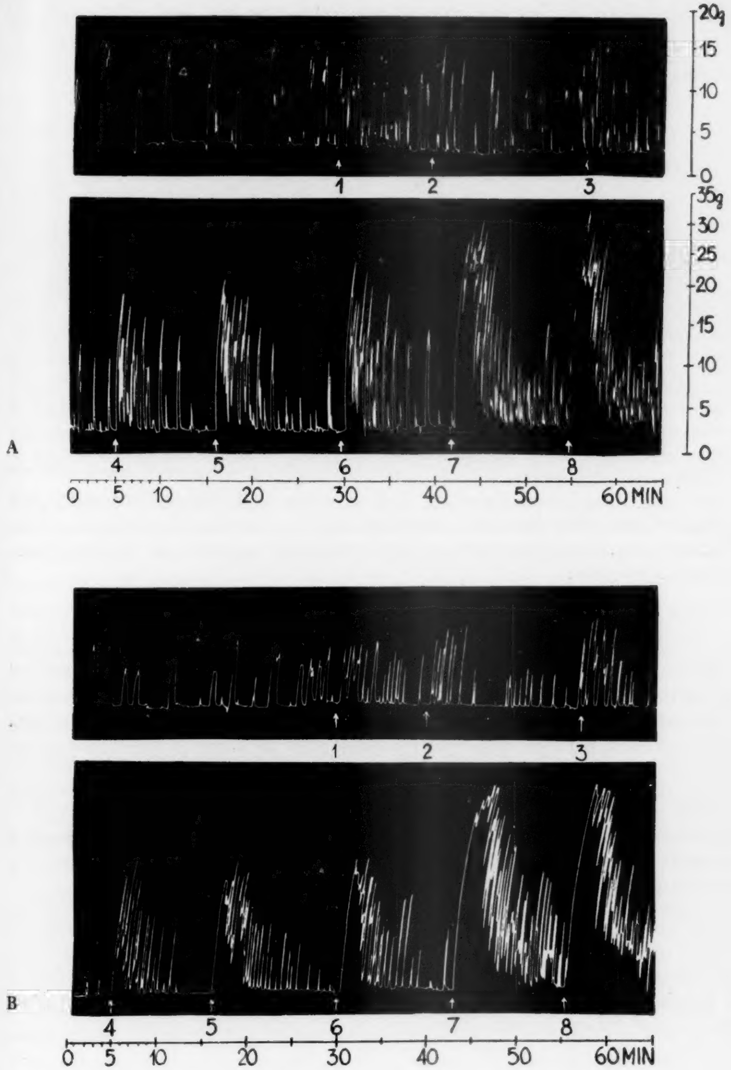

Fig. 1 A and B. Simultaneous isometric and isotonic recording of the effect of increasing doses of oxytocin in oestrogen-treated virginal rabbit in vivo. A. Isometric recording; weight of uterine segment 2.42 g. B. Isotonic recording; uterine segment 2.52 g; load 1.0 g; magnification 1 : 3.33.
1) 0.005 i. u. intravenously. 2) 0.01 i. u. 3) 0.02 i. u. 4) 0.05 i. u. 5) 0.10 i. u. 6) 0.20 i. u. 7) 2.0 i. u. 8) 5.0 i. u.

Table I. Threshold doses of methylergometrine and doses giving a non-repeatable response. Oestrogen-treated virginal rabbits

Dose in mg per rabbit	Number tested	Threshold response in	Non-repeatable response in
0.04	23	0	0
0.08	21	0	0
0.10	20	0	2
0.15	15	0	0
0.20	13	3	3
0.40	5	0	1
1.00	4	4	0

The mesometrium is left intact, and since the vessels are running at right angles to the uterus, the blood circulation of the segment is not disturbed, a fact which can easily be ascertained through the transparent chamber, in which the uterus is kept in a warm and moist atmosphere.

In some cases a double chamber was used, in which equal lengths of both uterine horns could be fixed. One horn was connected to an isometric myograph and the other to an isotonic lever. Recordings with both methods could then be made simultaneously on the same kymograph.

Material

White Land Rabbits of uniform breed, obtained from the State Serum Institute breeding farm, were used. Immature virginal rabbits weighing 2.7 to 3.0 kg were treated with 10 μg oestradiol benzoate (Ovex®, Leo) daily for seven to ten days before the day of experiment. Primiparous animals were used for experiments in the puerperium.

The rabbits were anaesthetized with an initial dose of 1.5—2.0 ml of Nembutal-sodium® (six per cent) intravenously, and the anaesthesia was maintained with intramuscular injections of 0.5—1.0 ml every 30 to 60 minutes during the experiment which could be extended up to eight hours.

The operating table was heated if necessary to maintain the body temperature which was controlled rectally. To obtain free respiration, tracheotomy with insertion of a cannula was performed in most experiments.

The substances to be tested were administered intravenously in the ears. The preparations were: pituitary oxytocin, partly a natural extract with a very low content of vasopressin (Pitupartin®, Alfred Benzon), partly a synthetic preparation (Syntocinon®, Sandoz), methylergometrine (Methergin®, Sandoz), and dihydroergotamine (DHE, Sandoz). Dilutions were made with saline, and the volume injected varied between 0.2 and 0.5 ml in most cases.

Results

A. *Oestrogen-treated virginal rabbits*

Spontaneous motility was recorded in each experiment as a control of the normal condition of the uterine muscle, and after each injection time was allowed to elapse until the spontaneous motility again showed the normal pattern. In

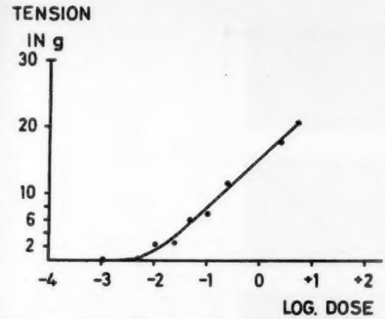

Fig. 2. Response to oxytocin in oestrogen-treated virginal rabbits plotted against log of dose. Isometric recording; tension developed over that of spontaneous contractions.

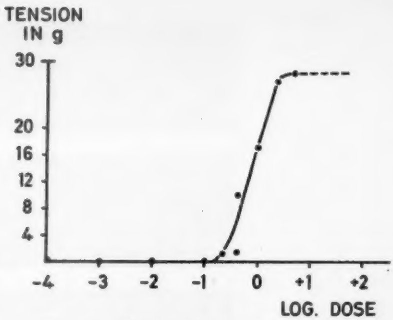

Fig. 3. Response to methylergometrine in oestrogen-treated virginal rabbits, expressed as tension developed over that of spontaneous contractions against log of dose.

control runs without administration of any drugs this pattern remained unchanged for several hours.

The tension developed both in spontaneous and induced contractions is very sensitive to changes in the body temperature which tends to fall in the anaesthetized animal. A fall to 36.5° C results in a marked decrease in sensitivity and tension development, but after restoration of the body temperature to the normal 39° C by heating the tension rises to previous values.

Pituitary oxytocin. The sensitivity to oxytocin was measured by determination of the threshold dose in ten rabbits. The threshold dose varied between 0.005 and 0.05 i. u., being 0.01 or 0.02 in the majority of the cases (Fig. 5). In four rabbits the threshold dose was determined both for natural and synthetic oxytocin and identical values were found.

The effect of increasing doses of oxytocin is illustrated on Fig. 1 A and B. The uterine muscle *in vivo* responds to low doses by increase of either tension or frequency of contractions, while higher doses increase both tension, frequency and duration. This makes it difficult to obtain a quantitative measure for the intensity of response, but if only the tension developed over that of the spontaneous contractions is considered a dose-response curve like Fig. 2 is obtained.

Methylergometrine. The sensitivity to this substance was determined in the same way. In every experiment the reactivity of the uterus was controlled with threshold doses of oxytocin (0.02 i. u.). In four experiments doses increasing from 0.001 mg up to 4 mg methylergometrine were given, in ten others up to 0.4 mg, because in the beginning only commercial solutions containing 0.2 mg/ml were available and it was considered undesirable to inject too large amounts. The results are summarized in Table 1.

No definite threshold dose could be established for methylergometrine.

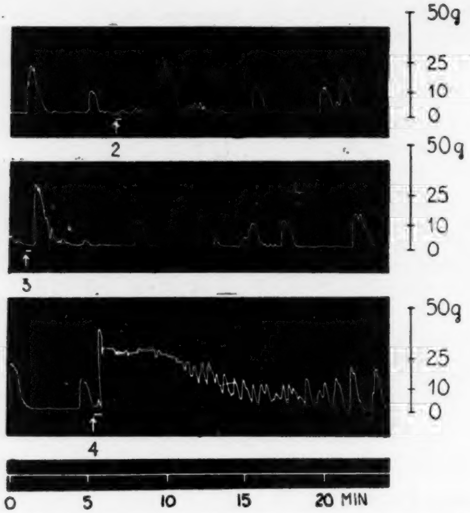

Fig. 4. Isometric tension development in oestrogen-treated virginal rabbit uterus *in vivo* in response to increasing doses of methylergometrine. Weight of uterine segment 1.55 g. 2) 0.04 mg; 3) 0.4 mg; 4) 4.0 mg methylergometrine intramuscularly.

Occasionally responses to doses of 0.1 mg, 0.2 mg and 0.4 mg occurred, but they were often vague and rarely repeatable. When 1.0 mg and more was administered the response was generally clear and repeatable. When the dose was further increased to 4.0 mg, maximal tension developed and the uterus often went into contracture for 5—10 min (Fig. 4, injection no. 4). In Fig. 3 the tension developed over that of spontaneous contractions is plotted in the same manner as for oxytocin.

Doses giving maximal tension affect the whole animal, the body is trembling and the rate of respiration is increased. One rabbit receiving a total of 5.0 mg methylergometrine died within fifteen min after the last injection.

If oxytocin has been administered before methylergometrine the sensitivity to the latter seems to be augmented.

In several cases the spontaneous activity of the uterus was adversely affected by methylergometrine. In three rabbits spontaneous activity stopped almost completely after 0.2 mg, and in several instances it disappeared for five to ten minutes after 0.2 or 0.4 mg. At the same time the sensitivity to oxytocin was often reduced, but it was restored later in the experiment.

Dihydroergotamine (DHE) was given to ten rabbits in doses of 0.5 or 1.0 mg. In two cases some inhibition of spontaneous activity was noticed. In one instance a slight oxytocic effect was seen after administration of 1.0 mg. In the doses mentioned dihydroergotamine did not influence oxytocin-induced contractions, whether given before or after the oxytocin.

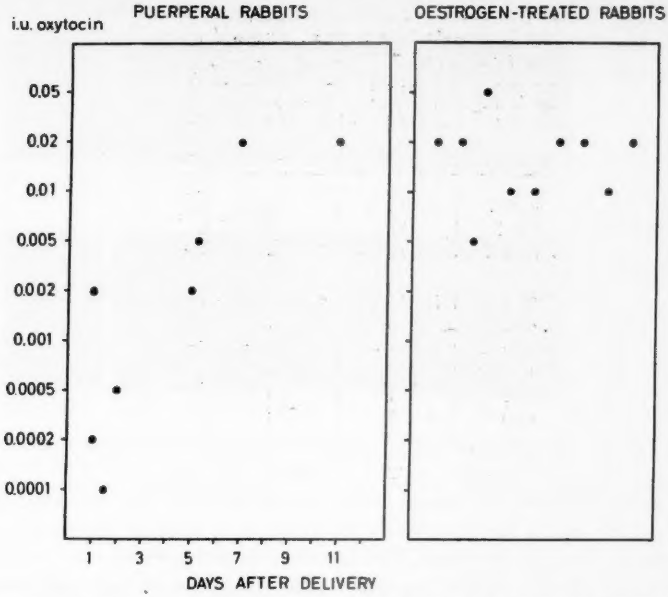

Fig. 5. Threshold doses for oxytocin in puerperal rabbits and in oestrogen-treated virginal rabbits.

B. Puerperal rabbits

Pituitary oxytocin. The sensitivity of the rabbit uterus to oxytocin is much increased immediately after delivery. The threshold dose 22 hours post-partum was found to be 0.0002 i. u. The sensitivity diminishes during the following days and on the fifth or seventh day post-partum it is the same as in the oestrogen-treated virginal animals, *i. e.* 0.02 i. u., as indicated in Fig. 5.

Methylergometrine. The threshold dose for this drug was not lower in the puerperal rabbits than in the oestrogen-treated animals (Table 2), but the response to the same dose was stronger and lasted much longer. In one experiment a lowered threshold was observed, 0.001 mg giving a slight effect. However, 0.1 mg was needed to repeat the effect. No other puerperal rabbit reacted to less than 0.2 mg methylergometrine, most of them required over 0.4 mg.

Dihydroergotamine. No effect of doses of 0.5 and 1.0 mg DHE could be observed in the puerperal rabbits except in one case where 0.5 mg seemed to inhibit spontaneous motility.

C. Simultaneous isometric and isotonic recording

In order to compare the information to be gained by these two methods both uterine horns of the rabbit were used in the same experiment, one for isometric and one for isotonic recording.

Table II. Threshold doses of methylergometrine and doses giving a non-repeatable response. Puerperal rabbits 1—11 days after delivery

Dose in mg per rabbit	Number tested	Threshold response in	Non-repeatable response in
0.0001	12	0	0
0.001	12	0	1
0.01	12	0	0
0.10	12	1	0
0.20	10	0	1
0.40	6	1	1
1.00	4	0	1
1.75	1	0	0
2.00	1	1	0
4.00	1	1	0

It was impossible to obtain good recordings with an unloaded isotonic lever which would be expected to show the most marked difference between the two methods of recording. The load had to be increased to approximately the weight of the uterine segment before good recordings were obtained, *i. e.* to one or two g. With this load isotonic recording gave the same results as the isometric recording, as shown by Fig. 1 A and B.

Discussion

It has been found that under oestrogen domination the rabbit uterus *in vivo* responds differently to increasing doses of oxytocin and methylergometrine. The ratio of the threshold dose to the dose causing maximal tension development in the contractions is for oxytocin between 1 : 1,000 and 1 : 100 and for methylergometrine 1 : 20 to 1 : 4, as can be seen by comparing the log dose-response diagrams for the two substances, the curve for methylergometrine having a much steeper slope (Fig. 2 and 3).

Furthermore, the duration of the effect of large doses of methylergometrine is much longer than that of large doses of oxytocin. Previous administration of oxytocin makes the uterus more sensitive to methylergometrine, while larger doses of the latter reduce the sensitivity to oxytocin.

The hormonal status of the rabbit has a great influence upon the sensitivity of the uterus to oxytocin, but this does not appear to be the case with methylergometrine. In the rabbit oestrogen increases the myometrial sensitivity to oxytocin, while progesterone reduces it (Knaus 1926, Robson 1933, 1937, Tripod and Meier 1948, Bengtsson 1957, and Schofield 1957). There is no systematic study of the sensitivity to methylergometrine during pregnancy

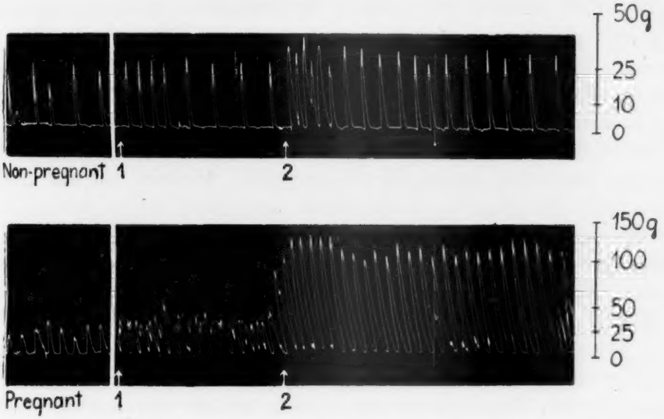

Fig. 6. Simultaneous isometric recording of the two horns of a unilaterally pregnant rabbit 22 hours post partum.
1) 0.005 i. u. oxytocin intravenously; 2) 0.05 i. u. Note the different scales of tension.

or during progesterone treatment, but existing observations indicate that it is not markedly diminished. KIRCHHOF *et al.* (1944) found *in vitro* greater sensitivity of the uterus of the 20 days pregnant than of the non-pregnant rabbit. BERDE and ROTHLIN (1953) got good effect *in vivo* with 0.2 mg/kg rabbit on the 22nd day of pregnancy, at which stage the sensitivity to oxytocin is still very low. The present study shows that immediately after delivery and in the first days thereafter, when the sensitivity to oxytocin is much increased, the threshold for methylergometrine is not lowered, although the uterine response is stronger as compared with the non-pregnant rabbit. Methylergometrine is thus more like histamine or adrenaline, to which the sensitivity of the uterus is not influenced by the ovarian hormones (TRIPOD and MEIER 1948, SCHOFIELD 1957).

In her studies of the influence of ovarian hormones on the rabbit uterus SCHOFIELD (1957) produced unilateral pregnancies experimentally and used the non-pregnant horn for experimentation. Immediately after delivery she found no appreciable increase in sensitivity to oxytocin over that encountered in the non-pregnant rabbit. This seems to be in disagreement with our findings, but in one instance we happened to find a rabbit in which one horn had been pregnant and the other not. With simultaneous recording we found the sensitivity of the pregnant horn to be greater than that of the non-pregnant horn 22 hours after delivery. Later unilateral pregnancies were produced experimentally and the finding could be confirmed; especially the intensity of the response was much greater in the pregnant horn (Fig. 6). Such a difference between the two horns in a unilateral pregnancy supports the concept of a

direct, localized effect of the product of conception upon the myometrium (CSAPO 1956 a).

Another difference in the action of oxytocin and methylergometrine is illustrated by a series of experiments in which it was attempted to induce labour in rabbits the last two days before term (FUCHS and FUCHS 1958). Thirty days after mating 26 of 58 rabbits delivered their litter on 1.0 i. u. oxytocin, and of the remaining almost all (94 per cent) delivered on the same dose 24 hours later. The majority of the rabbits were given 0.1 or 0.2 mg methylergometrine and some even 1.0 or 2.0 mg thirty minutes before the oxytocin, but only one delivered on methylergometrine thirty days after mating, and then only one of the foetuses, and another one delivered its litter thirty-one days after mating on methylergometrine.

Some light has been shed on the mechanism of the action of oxytocic substances by experiments of CSAPO (1954, 1956 b), COUTINHO (1959), and BÜLBRING (1959). These experiments seem to indicate that oxytocic substances act either by altering the permeability of the muscle cell membrane or as carriers of ions. Much more experimentation is needed, however, before the mechanism is fully clarified.

Dr. A. CSAPO, of The Rockefeller Institute of New York, we thank for his cooperation in the early phase of this work, and for stimulating discussions on uterine physiology. The experiments were carried out at the Department of Medical Physiology and the Department of Hygiene of the University of Copenhagen, and we thank Prof. E. LUNDSGAARD and Prof. P. BONNEVIE for excellent working facilities. The work has been supported by a grant from the Nordisk Insulinfond and we are also indebted to Sandoz A. G., Basle, for support and the supply of Methergin and Syntocinon.

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From the Institute of Zoophysiology, University, Uppsala, Sweden

The Histamine Methylating Enzyme System in Liver

By

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Abstract

LINDAHL, K. M. *The histamine methylating enzyme system in liver.* Acta physiol. scand. 1960. *49.* 114—138. — An enzyme is described which catalyses the transfer of the methyl group of S-adenosylmethionine to histamine at the imidazole nitrogen remote from the side chain. A method for the assay of the enzymic activity is described. The enzymic activity is enhanced with increasing pH. The influence of certain inhibitors examined suggests that the enzyme has essential sulfhydryl groups. Studies on the intracellular distribution indicate that the enzyme is localized to the soluble cytoplasm. The enzyme has been found in the liver of mouse, pig, hamster, rabbit, cat, and man.

It is well known that histamine occurs naturally in most tissues, and that this amine is regularly transferred to the blood, is metabolized, and excreted in the urine.

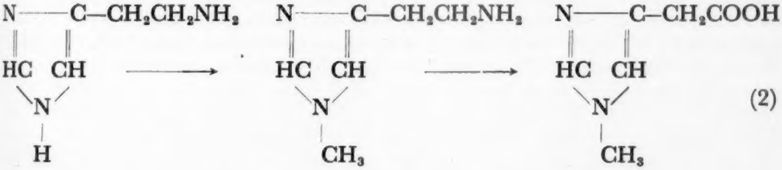
For many years diamine oxidase (or histaminase)[1] was the only enzyme known to attack histamine. Its physiological role as well as its biochemical properties have been thoroughly examined (for references see ZELLER 1952, TABOR 1954, LINDELL 1958). According to the generally accepted view the products of histamine deamination and oxidation are imidazole acetaldehyde, ammonia, and H_2O_2:

$$RCH_2CH_2NH_2 + O_2 + H_2O \rightarrow RCH_2CHO + NH_3 + H_2O_2 \qquad (1)$$

[1] The following abbreviations will be used: DO: diamine oxidase. ImAA: imidazole-4(5)-acetic acid. MeImAA: 1-methylimidazole-4-acetic acid methylhistamine: 1-methyl-4-(β-amino-ethyl)imidazole. AMe: S-adenosylmethionine. TCA: trichloroacetic acid. GSH: reduced glutathione. ATP: adenosine triphosphate. EDTA: ethylendiaminetetraacetic acid (Versene). cpm: counts per minute. IBINH: 1-isobutyl-2-isonicotinylhydrazine. pCMB: para-chloromercuribenzoate. Tris: 2-amino-2-hydroxymethyl-1,3-propandiol.

The aldehyde has never been isolated, but its formation is indicated by the accumulation of ImAA on further oxidation (TABOR and BAUER 1951, LINDAHL *et al.* 1958). It has also been chromatographically and chemically demonstrated by KAPELLER-ADLER and FLETCHER (1958).

Recently a series of papers by SCHAYER and his collaborators introduced a new conception of histamine metabolism (SCHAYER 1952, 1953, SCHAYER, KENNEDY, SMILEY 1953). Their experiments utilized isotope technics and radioactive histamine for the study of the metabolism *in vivo*. After injection of C^{14}-labelled histamine the urine was collected, and analyzed by paper chromatography. Three radioactive areas were obtained, one representing histamine and acetylhistamine, and two representing unknown metabolites. The distribution of the radioactivity between these areas was affected differently by aminoguanidine and iproniazid. These results together with other evidences led the authors to postulate two ways for the degradation of histamine. Later the two unknown metabolites have been identified as ImAA riboside, the final product of the action of DO, and MeImAA (BOUTHILLIER and GOLDNER 1953, KARJALA 1955, TABOR and HAYAISHI 1955, KARJALA and TURNQUEST 1955). The postulated enzyme system leading to the formation of MeImAA was called "Histamine metabolizing enzyme II". The *in vivo* findings demonstrated that the system in question was a combination of methylating and oxidative enzymes which brought about the following reaction steps (SCHAYER 1955, SCHAYER and KARJALA 1956, SCHAYER 1959):



(2)

Histamine methylhistamine MeImAA

MeImAA has been shown to be present in the urine of the majority of the studied species (SCHAYER and COOPER 1956, SCHAYER 1956). This fact stresses the great importance of this route of histamine metabolism. It was therefore highly desirable to make an active enzyme preparation available for biochemical studies. KOBAYASHI and SCHAYER reported in 1956 that they had found an enzymatic formation of MeImAA in cat liver homogenates (KOBAYASHI and SCHAYER 1956, KOBAYASHI 1958). In mouse liver KOBAYASHI failed to demonstrate the existence of the methylating enzyme system (1957). However, he found that in this material the added histamine was oxidized to ImAA by an unknown enzyme with properties similar to those attributed to monoamine oxidase. The author did not try methylhistamine as a substrate for this oxidase, but in the light of later results (ROTHSCHILD and SCHAYER

1958) it might be worth while to reinvestigate whether or not this enzyme is able to catalyse the second step in reaction (2).

The present paper deals with the enzyme system that catalyses the methylating step in reaction (2). The starting point for the investigations was the discovery that, although mouse liver homogenates did not form MeImAA, mouse liver slices certainly were capable of catalyzing the process (LINDAHL 1958 a). A fractionation of the homogenate was performed leading to an enzyme preparation which, in the presence of suitable cofactors, was capable of transforming histamine to methylhistamine (LINDAHL 1958 b). No oxidation occurred. It was shown that the methyl group of methionine was utilized as methyl donor, and that AMe (CANTONI 1955) had to be an intermediate in this transmethylation reaction (LINDAHL 1958 c, 1958 d). The requirement for AMe has later been demonstrated also by BROWN, AXELROD and TOMCHICK (1959).

The enzyme, called histamine methyl-transferase, was further purified, and studied with respect to substrate specificity and affinity, pH dependency, and certain inhibitors. Also its occurrence and localization in the cell were studied.

Material and methods

Preparation of AMe. AMe was prepared from bakers' yeast according to SCHLENK and DE PALMA (1957) and SCHLENK, DAINKO, and STANFORD (1959). The obtained product was analyzed on paper chromatograms developed in ethanol-water-acetic acid (65:34:1, v/v). The spots were located with ultraviolet light. The Rf values obtained were identical with those reported by PARKS and SCHLENK (1958). The hydrolysis of AMe with 0.1 N NaOH was followed in a Beckman spectrophotometer DK. 2. Within 10 min the absorption at 250 mμ had dropped to 54 per cent of the original value, indicating the hydrolysis of the glycosidic bond under formation of adenine. This value indicates that the preparation does not contain contaminating adenosine or alkali resistant derivatives. Alkaline hydrolysis with brief heating yields methionine which was identified by chromatography. The ribose component was detected by spraying with ammoniacal solution of $AgNO_3$ (PARTRIDGE 1948).

Preparation of labelled methylhistamine. Labelled methylhistamine was prepared by enzymatic methylation of C^{14}-histamine, AMe was added in excess, and the incubation prolonged until practically all added histamine was methylated. The reaction product was purified by extraction into chloroform after addition of NaOH to pH 13 and saturation with Na_2SO_4. An XE 64-column in H^+-form was prepared, and washed in acetone, NaOH, and HCl, according to HIRS (1955). Finally it was washed with acetone—water and acetone. The chloroform was poured directly on the column, and washed out with acetone and water. The elution was performed with hydrochloric acid, 1 ml fractions being collected and analyzed for radioactivity. The portion containing methylhistamine was analyzed by paper chromatography, sometimes after partial lyophilization. It was found to be contaminated with a small amount of an unknown ninhydrine reactive substance. When enough material was spotted on the chromatogram to make the methylhistamine itself detectable with ninhydrine reagent, a spot appeared above the radioactive material. No radioactive contaminations could be detected when at least 50 mμC methylhistamine was chromatographed with carrier histamine. With this method 0.5 mμC C^{14}-histamine would give

a discernible peak. Thus the methylhistamine is contaminated with less than 1 per cent histamine.

Assay of histamine methyltransferase by extraction of methylhistamine with chloroform. Starting from the difference, demonstrated by ROTHSCHILD and SCHAYER (1958), in the distribution coefficient for histamine and methylhistamine in the system alkaline salt solution—chloroform a method for the determination of the histamine methylating ability in enzyme extracts has been developed.

The enzyme preparation is incubated in test tubes with C^{14}-histamine and other additions in a total volume of 200 μl. The incubation is interrupted by the addition of 10 μl 1 M $CuCl_2$ solution.[2] Subsequently 100 μl 10 N NaOH, saturated with Na_2SO_4 are added under cooling. Now 1 ml chloroform is added, the tube is closed with aluminium foil and a rubber band, shaken for 10 min in a shaker, and afterwards centrifugated for 5 min. The diameter of the tube has been chosen so that a light touch of the surface makes the water film burst, the water phase being collected close to the walls. Now samples can easily be sucked up without risk of contamination. For the determination of C^{14} pressed trays of sheet aluminium have been used. 100 to 200 μl of the chloroform phase are plated together with 5 μl of 0.1 % picric acid in alcohol (w/v), and allowed to dry. Between 25 and 250 μl have been put upon a plate, and no self-absorption could be observed. The samples have been counted under an end window Geiger tube (20th Century Electronics EW 3 H) with a diameter of the window of 23 mm. The plate has a diameter of 12 mm, and the distance between sample and window is 5 mm. From each incubation tube 3 plates are prepared, and at least 2,500 impulses are counted for every plate. The obtained average value is proportional to the quantity of methylhistamine in the way that can be seen from the standard curve in Fig. 1. The latter has been obtained by analysis in the above described way of known quantities of C^{14}-methylhistamine and C^{14}-histamine with a constant total quantity of radioactivity.

A straight line, described by the equation

$$y = x\,\frac{(a\text{-}b)\,c}{10^4} + \frac{bc}{10^2} \tag{3}$$

is obtained, where a = per cent methylhistamine that passes into the chloroform, b = per cent histamine that passes into the chloroform, and c = cpm for the total quantity of added C^{14}. The purity of the enzyme preparations and other additions can to a certain extent influence the extraction with chloroform of both histamine and methylhistamine. For this reason it is necessary to carry out for every experiment control determinations of the efficiency of the extraction at 0 and 100 % methylhistamine, respectively. By means of these values it is then possible to calculate from (3) the methylhistamine content of the unknown samples in per cent of the added histamine.

The reliability of the method was tested by the simultaneous performance of 27 identical incubations. For each of them the methylhistamine formed was determined as above. The mean value for the radioactivity obtained in 100 μl chloroform was 336 cpm, the standard deviation being 7.9. In another series on 16 samples the mean value was 220 cpm, the standard deviation being 4.9.

[2] This method of stopping the reaction was used in all experiments, where only the enzyme and the two substrates were present. Whenever foreign components were included, the reaction was interrupted, at least as a control, by addition of 10 μl conc. HCl, and the tubes inserted in boiling water for 30 sec before addition of NaOH, evaporation being prevented by a cooling coil. This precaution has to be taken in view of the ability of copper to react with many agents. However, it makes the withdrawal of chloroform samples somewhat more delicate. In other respects no differences are entailed.

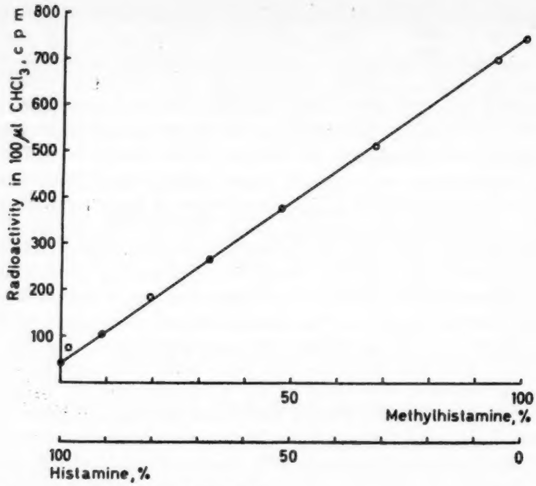


Fig. 1. *Standard curve on analysis of methylhistamine.*
C^{14}-histamine and C^{14}-methylhistamine in varied proportions, together 0.05 μC and appropriate amounts of other additions were treated as the unknown samples. The figure shows the radioactivity obtained in the chloroform layer, correlated to the added amount of methylhistamine and histamine.

Quantitative analyses of C^{14}-labelled histamine and its metabolites was performed with the isotope dilution technique developed by Schayer and his colleagues (Schayer and Karjala 1956, Schayer and Cooper 1956). The standard technique employed is as follows:

Histamine: 66.4 mg histamine dihydrochloride (or multiples of it) are dissolved, and mixed with an appropriate amount of the sample to be analyzed. The sample is shaken gently for 10 min. TCA is added to give a final concentration of 8 %. After at least 1 hr in the cold the precipitate is filtered off, and the TCA removed by ether extraction. The filtrate is evaporated to dryness, and redissolved in 2 ml of water. 180 mg picric acid in 2 ml alcohol are added. The solution is heated, active carbon is added, and the mixture filtered through Celite. The crystallization of histamine dipicrate starts on scratching the glass vessel containing the hot filtrate. Before being collected and washed the crystals are allowed to settle overnight in the cold. The picric acid is removed by passing the histamine dipicrate dissolved in hot water through a column of Dowex-1 in Cl^--form. The eluate is evaporated and dissolved in 2 ml water, 280 mg p-iodobenzenesulphonyl (pipsyl)[3] chloride in 3 ml dioxane and 200 mg solid $NaHCO_3$ are added. The mixture is shaken mechanically for 30 min. Upon gradual addition of water the crystallization starts. After its completion the crystals are collected, and washed with 25 % alcohol, redissolved in acetone, and treated with active carbon. After filtration through Celite, pure crystals are obtained by gradual addition of water to the filtrate. The crystallization from acetone is repeated with the use of different types of active carbon until the radioactivity remains constant during three successive crystallizations.

[3] *Iodobenzenesulphonyl chloride* was synthesized at the Department of Organic Chemistry, Uppsala, under the supervision of Dr. L. Schotte and, later, of Dr. G. Claeson. To both of them I am under great obligation.

ImAA[4]*:* 95 mg carrier ImAA-hydrochloride is added to the sample. The deproteinized sample is acidified, and passed through a Dowex-50-H^+ column. The column is washed thoroughly with hydrochloric acid and water. The ImAA is eluted with NH_4OH. After evaporation picrate and, finally, the pipsyl derivative is prepared as described for histamine, with the exception that the pipsylated sample has to be neutralized with acetic acid before the crystallization starts.

MeImAA[4]*:* 99 mg carrier MeImAA are added, and crystals of the picrate prepared as described for ImAA.

Methylhistamine[4]*:* 71 mg carrier methylhistamine are added to the sample. The proteins and, thereafter, TCA are removed as above. By addition of 10 N NaOH the filtrate is made strongly alkaline. Solid Na_2SO_4 is added to saturation. The solution is extracted with chloroform which is evaporated to dryness after addition of picric acid as above. The residue is dissolved in alcohol, treated with carbon, and methylhistamine dipicrate is repeatedly crystallized to give constant radioactivity.

Measurement of radioactivity was carried out in a window-less flow counter (Tracerlab. SC-16) background 31 cpm. Each sample was at least counted for 5 × 6 min, alternatively until 10,000 counts had been obtained. A C^{14}-standard was counted for every fourth sample and background was counted twice a day.

The crystals are mounted on plates 25 mm wide and 5 mm high, and measured at infinite thickness. From the third crystallization and onwards the plates are weighed. If the amount of radioactive material is too small to permit counting at infinite thickness, it is accurately diluted, and the observed count multiplied by a correction factor. All values are given as cpm above background.

Protein determination. The protein concentration was estimated from the light absorption at 260 and 280 mμ according to WARBURG and CHRISTIAN. The values obtained agreed rather well with controls performed with the biuret method. The latter method was used for protein determinations in homogenates and other fractions that were turbid solutions. Both methods were employed as described by LAYNE (1957).

Ascending paper chromatography with t-butanol-formic acid-25 % pyridine in the proportions 70:15:15 for development, has been used to identify histamine and its metabolites. The chromatograms were run for 18 hours at a temperature of 24° C. Test substances were made visible by diazotization as described by TABOR (1957) or by dipping the paper in 0.5 % ninhydrine in acetone. The radioactivity was assayed in an automatic scanning device (Friesecke-Höpfner, FH 452 with methanflow tube FH 407). When pure compounds are chromatographed 0.5 mμC gives a broad peak of about 7 mm height, the background peaks not exceeding 4 mm.

Experimental

Enzyme preparation

In a previous investigation it has been shown that methylation of the ring nitrogen of histamine remote from the side chain may take place *in vitro* in the mouse liver.

Slices of mouse liver were incubated in Krebs-Henseleit buffer with radioactive histamine, methionine, and glucose. An enzymatic formation of MeImAA

[4] *Nonradioactive ImAA, MeImAA and methylhistamine* were obtained from Dr. R. W. Schayer, Merck Institute for Therapeutic Research, Rahway, N. J., as is hereby gratefully acknowledged. Small amounts were also synthesized according to the methods of Bauer and Tabor (1957) and Rothschild and Schayer (1958). Highly purified ImAA, used as test substance in chromatographic analyses, was kindly supplied by Dr. H. Tabor.

Table 1. Methylhistamine synthesis in mouse liver preparations

The incubation mixture contained 0.01 mmole methionine, 0.012 μmole C^{14}-histamine, 2.5 μmoles $MgCl_2$, 0.5 μmole ADP, 0.1 μmole ATP, 8 μmoles α-ketoglutarate, 0.5 ml enzyme preparation as described in text, and 0.05 M Tris buffer pH 7.4 to a total volume of 1 ml. Incubation at 37° for 120 minutes.

Enzyme preparation	Protein content of enzyme preparation, mg/ml	Per cent of radioactivity recovered	
		as histamine	as methylhistamine
Homogenate	75	71	17
Supernatant I	28	62	38
Supernatant II	18	31	71

was demonstrated by chromatographic methods. Under corresponding circumstances finely ground mouse liver catalyses methylation of histamine only very slowly.

In order to increase the specific activity the following procedure for fractionation was applied:

Grinding in 0,1 M NaAc-buffer pH 5,6
↓
Homogenate
↓
Centrifugation 25,000 × *g*
↓
Precipitate discarded ← Supernatant I
↓
47° C, 8 min.
↓
Precipitate discarded ← Supernatant II

The methylating ability of the active fractions can be seen in Table I. The methylation has been determined with the isotope dilution method. The fractionation procedure has been examined also with chromatographic methods, the results being accounted for in an earlier publication (Lindahl 1958 b).

Table I shows that methylhistamine is the only product of the histamine metabolism when enzyme preparations sup. I and II are used. It is therefore possible after these purification steps to determine the activity of an enzyme preparation by the quantitative analysis of the methylhistamine formed, either by the isotope dilution method or by the chloroform method.

The origin of the methyl group. Experiments with continued fractionation of sup. II resulted in entirely inactive fractions. The inactivation during the

Table II. AMe as methyl donor to histamine

The incubation mixture contained 0.084 μmole C^{14}-histamine, 0.5 ml enzyme preparation, one of the following combinations, and 0.5 M Tris, pH 7.4 to make 1 ml.
1) 3.2 μmoles ATP, 50 μmoles $MgCl_2$, 6.7 μmoles methionine, 0.82 μmole GSH.
2) 1.5 μmoles AMe.
Incubation for 120 min at 37° C. The histamine disappearance is determined, with the isotope dilution method, as histamine recovery for the homogenate, and as methylhistamine formation for "sup. II". 0.084 μmole C^{14}-compound here corresponds to 6,130 cpm.

Enzyme preparation	Incubation conditions	Histamine, cpm	Methyl-histamine, cpm	Histamine metabolized, %
Homogenate	1	4,178		32
Homogenate	2	32		100
Supernatant II	1		1,663	27
Supernatant II	2		6,183	100

fractionation was assumed to derive from the inadequacy, at the assay, of the medium for the purified enzyme. A search for more efficient methyl donors was obviously necessary.

The use of methionine labelled with C^{14} in the methyl group[5] has shown this substance to function as methyl donor (LINDAHL 1958 c): 0.5 ml of a dialyzed enzyme preparation "sup. II" was incubated for 2 hrs with 1) 0.084 μmole C^{14}-histamine and 0.67 μmole nonradioactive methionine and 2) 0.084 μmole nonradioactive histamine and 0.67 μmole C^{14}-methionine respectively. The medium contained in addition 7.5 μmoles ATP, 50 μmoles $MgCl_2$, and 0.8 μmole GSH in 0.5 M Tris buffer pH 7.4 to the volume 960 μl. Labelled methylhistamine was in either type of incubate determined by the isotope dilution method. In incubate no. 1 with C^{14}-histamine 0.034 μmole of the labelled material was recovered as methylhistamine, and in the C^{14}-methionine incubate no. 2 the methylhistamine count corresponded to 0.0096 μmole, provided the specific labelling being the same as that of the added C^{14}-methionine. The experiment shows that about 1/3 of the methylhistamine formed has obtained the carbon in the methyl group from the added C^{14}-methionine.

If thus the methionine can act as the primary methyl donor, the purification experiments indicate that the methionine cannot be exploited as such by the histamine methylating enzyme, but that under the above experimental conditions components in the impure enzyme preparation have formed a methionine derivate that makes possible the transfer of the methyl group of the methionine to the histamine.

For this reason the above mentioned additions were replaced by AMe. The methylation of the histamine was then measured with the isotope dilution method.

[5] C^{14}-methionine, homocysteine and betaine were generous gifts from Dr. L. E. Ericson, Royal Institute of Technology, Stockholm.

Table III. Fractionation of mouse liver homogenate

0.4 ml enzyme preparation is incubated for 1 hr with 0.084 μmole C^{14}-histamine, 1.5 μmoles AMe and 0.5 M Tris pH 7.4 to make 1 ml.

Enzyme preparation	% of radioactivity recovered as methylhistamine	mg protein/ml of enzyme preparation
Homogenate	68	78
"Sup. II"	80	15.7
0—25 % $(NH_4)_2SO_4$	—	0.5
25—47 % $(NH_4)_2SO_4$	16	7.3
47—68 % $(NH_4)_2SO_4$	77	6.3
Final supernatant....	—	1.5

The results (Table II) show that the homogenate and the enzyme preparation "sup. II" are admittedly able to use methionine, but that an addition of AMe speeds up the enzyme reaction very considerably. It may be inferred that the synthesis of AMe is the limiting step.

In another experimental series (Lindahl 1958 d) both methylhistamine and the remaining histamine were analyzed after incubation of C^{14}-histamine with dialyzed "sup. II" without any further additions. If no AMe had been added, the entire radioactivity was recovered as histamine. In the presence of AMe histamine and methylhistamine taken together accounted for all the added radioactivity. Omission of the enzyme resulted in complete loss of histamine methylating activity as did boiling of the enzyme before the incubation.

In the following experiments AMe was included in the medium at the assay of the histamine methylation.

Fractionation of "sup. II" with $(NH_4)_2SO_4$. In preliminary experiments *mouse liver* was used for fractionation with $(NH_4)_2SO_4$. To 2 ml "sup. II" were added 0.84 g $(NH_4)_2SO_4$ in 3 equal portions. The precipitate formed upon each addition was removed by centrifugation after 15 min in an ice bath, and was dissolved in 1 ml 0.1 M NaAc buffer, pH 5.6. The solution was dialysed for two hours against 0.05 M NaAc buffer, pH 5.6, centrifugated, and diluted to 2 ml. The centrifugate remaining after the last precipitation with $(NH_4)_2SO_4$ was dialysed for 12 hrs prior to use. The formation of methylhistamine was determined by isotope dilution assay after incubation with AMe and C^{14}-histamine. The results are accounted for in Table III.

"Sup. II" obtained from *pig liver* completely transforms histamine into methylhistamine, if AMe is present in excess (Table IV). For this reason pig liver was used as raw material, when larger quantities of enzyme were to be prepared.

Table IV. Histamine methylation by pig liver enzyme

0.5 ml pig liver enzyme solution was incubated for 1 hr at 37° C with 0.084 μmole C^{14}-histamine, 1 μmole AMe and 0.5 M Tris buffer pH 7.4 to make 1 ml. Histamine and methylhistamine were determined with isotope dilution assay.

	Total C^{14} calculated	Histamine found	Methylhistamine found
cpm	6,130	558	5,581
%........	100	9.1	91

In order to establish the requirements of the enzyme, the fraction precipitated between 48 and 68 % $(NH_4)_2SO_4$ was used in the experiments accounted for below.

Elaboration of conditions requisite for the studies of the histamine transmethylation

Relation between substrate concentration and enzyme activity. The velocity of the histamine methylation at low concentrations of histamine was determined by use of C^{14}-histamine with high specific activity.

The formation of methylhistamine at initial concentrations of histamine from 7.25 μM to 422 μM and constant concentration of AMe during 10 min incubation was determined (Table V). The values obtained plotted in the double reciprocal manner gave a K_m for histamine of about 1×10^{-4} M. It must, however, be emphasized that the use of still lower histamine concentrations would be desirable. By addition of non-radioactive histamine the initial concentration was varied from 69 μM to 1.47 mM. The low specific activity makes the determination of methylhistamine in such experiments a rather delicate task. However, no definite tendency towards changed reaction velocity was observed.

Influence of methylhistamine on the methylation velocity. The influence of methylhistamine on the reaction velocity was studied in experiments in which non-radioactive methylhistamine was added to the incubates. *Fig. 2* shows the decrease in histamine methylation velocity on account of increasing concentrations of methylhistamine added at zero time.

Influence of the concentration of AMe on the methylation velocity. The concentration of AMe was varied from 0.15 mM to 7.5 mM, while the concentration of histamine was kept constant at 0.25 mM. The results are shown in Table VI. No attempt to determine K_m has been made.

Proportionality to the enzyme concentration. Under the experimental conditions used the histamine methylation was found to be proportional to the enzyme concentration provided that not more than about 50 % of the substrate were methylated during the incubation period (Fig. 3).

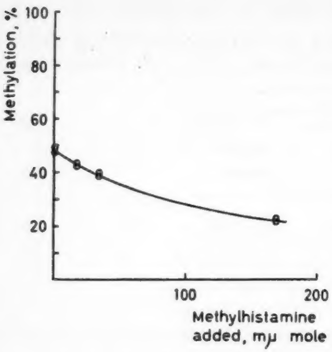


Fig. 2. *The influence of methylhistamine on the histamine methylation velocity*
Initial concentrations are 34 mμmoles C^{14}-histamine, 0.25 μmole AMe, 25 μl enzyme solution, and methylhistamine as indicated in 0.5 M Tris buffer pH 8 to a volume of 200 μl. Incubation time 30 min.

Time course of methylation of histamine. Standard conditions for assay. The progress of the methylation of histamine with incubation time has been studied in a series of experiments with varying concentrations of histamine and enzyme. Different preparations did not give identical progress curves, but very often a slight decline in methylhistamine formation with time was noticed even in the presence of an excess of substrate. However, such standard conditions were adopted for the following experiments as permitted the methylation to be considered approximately proportional to incubation time for 30 minutes (Fig. 4). With most enzyme batches favourable conditions were obtained, when 25 μl enzyme solution were incubated at 37° C with 0.034 μmole C^{14}-histamine and 0.3 μmole AMe in 0.5 M Tris buffer pH 8.0 to a total volume of 200 μl. The enzyme preparations used had a protein content of about 25 mg per ml. Under the standard conditions described above they catalysed the formation of about 0.05 μmole methylhistamine/mg protein/hr.

Table V. The relation between histamine concentration and methylhistamine formation
Every incubate contained 25 μl enzyme solution, 0.10 μmole AMe, 0.5 M Tris buffer pH 8.0 and C^{14}-histamine as indicated in a volume of 200 μl. Methylhistamine was determined with the chloroform method after 10 min. incubation at 37° C.

Specific activity	Initial histamine cencentration, μM	Methylhistamine formed, μM
7.25 mC/mM	17.25	9.3
	34.5	16.4
	86.4	28.5
	172.5	36
1.48 mC/mM	84.2	29.5
	168.4	40.5
	422	52.7

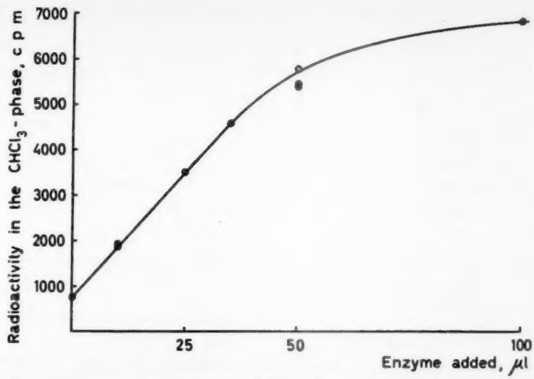


Fig. 3. *Relation between enzyme concentration and histamine methylation.*
Different volumes of enzyme solution were incubated for 30 min with 0.034 µmole C^{14}-histamine, 0.1 µmole AMe and 0.5 M Tris buffer pH 8 to the volume 200 µl. Methylhistamine was determined by the chloroform method. 50 % methylation corresponds to 3,780 cpm, 100 % to 6,800 cpm.

Relation between pH and enzymic activity

The enzyme preparation from pig liver was incubated with C^{14}-histamine and AMe in buffer solutions of the pH values indicated in *Fig. 5.* The methylhistamine formed was determined with the chloroform method. The methylation is accelerated with increasing pH.

In acid medium the enzymic activity is reversibly inhibited while the sudden inactivation in alkaline medium is irreversible (Table VII).

Specificity of the enzyme preparation

Of the methyl compounds investigated only AMe acted as a methyl donor to histamine. Betaine[5], methionine, or methionine sulfoxide could not replace AMe (Table VIII).

Nicotinamide, homocysteine[5], glycocyamine, histidine, adrenaline[6] and noradrenaline[6] were studied as possible methyl acceptors. These substances were included in the standard incubation medium, and their influence on the

Table VI. The influence of AMe on the methylation velocity

The incubates contained 50 µl enzyme solution, 0.05 µmole C^{14}-histamine, and 0.5 M Tris buffer pH 8.0 to a volume of 200 µl. Methylhistamine was determined with the chloroform method after 30 min incubation at 37° C.

AMe added, µmole	0.03	0.3	0.75	1.5
Histamine methylated, %	14.5	24	23	21.5

[6] *Adrenaline and noradrenaline* bitartrate were kindly supplied by Astra, Södertälje.

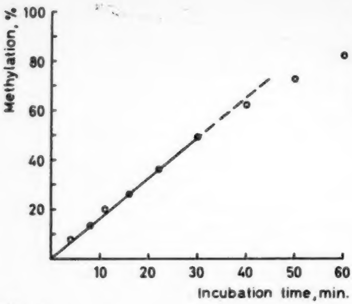


Fig. 4

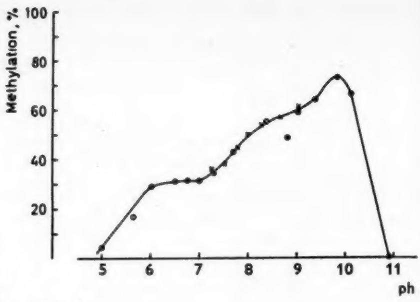


Fig. 5

Fig. 4. *Methylation of histamine with time.*
The incubate contained per 200 μl, 25 μl enzyme solution, 0.034 μmole C^{14}-histamine and 0.3 μmole AMe in 0.5 M Tris buffer pH 8, the total volume being 2 ml. Portions on 200 μl were withdrawn at intervals indicated, and the methylhistamine formed determined with the chloroform method.
Fig. 5. *The influence of pH on the histamine methylation.*
25μl enzyme solution is incubated with 0.034 μmole C^{14}-histamine, 0.25 μmole AMe, and 75 μmoles buffer in a volume of 200 μl. The pH values indicated refer to the added buffer. The final solution obtains the pH of the added buffer within 0.1 unit.
O—O PO_4-buffer, +—+ Tris-buffer, ●—● Glycine buffer.

methylhistamine formation as measured with the chloroform method was recorded at different concentrations. Under the conditions of the present experiments glycocyamine and nicotinamide were found without effect on the histamine methylation. Homocysteine and histidine had a slightly stimulating effect if any (Table IX). Adrenaline and noradrenaline in high concentrations, however, appeared to inhibit the formation of methylhistamine (Table X).

Table VII. The stability of histamine methyl-transferase in acid and alkaline medium

The incubations were performed under standard conditions at pH 8.0 for 30 min after 10 min preincubation of the components at acid or alkaline pH. The controls were complete incubations performed at the same pH as the preincubations.

pH of preincubation	Component preincubated	Histamine methylated, %
5.4	control	0
	AMe	49
	C^{14}	55
	enzyme	50
10.8	control	1
	AMe	58
	C^{14}	55
	enzyme	5

Table VIII. The relation of different methyl donors to histamine methyl-transferase

25 μl enzyme solution were incubated for 30 min at 37° C with 0.034 μmole C^{14}-histamine, the additions indicated and 0.5 M Tris buffer pH 8.0 to a volume of 200 μl.

	Chloroform-extractable radio-activity, cpm	Calculated histamine methylation, %
No additions	580	1.5
0.25 μmole AMe	3,820	51
0.25 μmole AMe + 0.25 μmole methionine	3,920	52.5
— 0.25 μmole methionine	560	1
— 1 μmole methionine	580	1.5
0.25 μmole AMe + 0.25 μmole methioninesulfoxide	3,930	52.5
— 0.25 μmole methioninesulfoxide	550	1
— 1 μmole methioninesulfoxide	520	0.5
0.25 μmole AMe + 0.25 μmole betaine	3,840	51
— 0.25 μmole betaine	530	0.5
— 1 μmole betaine	510	0

Inhibitors of histamine methylation

pCMB. The inhibition of the histamine methylation by pCMB is shown in Fig. 6. Concentrations from $5 \cdot 10^{-8}$ to $5 \cdot 10^{-3}$ M were tested. The inhibition is completely abolished by GSH in about equimolar concentration. (See also LINDAHL 1959).

In the above experiments pCMB was added directly to the incubation medium. In another type of experiment the enzyme alone was incubated with 0.005 M pCMB for 10 min at 37° C., and dialysed for 20 hrs against several portions of 0.5 M Tris buffer pH 8.0. A quantity of uninhibited enzyme was dialysed in the same manner. The pCMB pre-treated enzyme was still inactive, but the inhibition was counteracted by the addition of GSH. The complete removal of excess inhibitor was demonstrated as follows: a portion of the pCMB pre-treated and dialysed enzyme solution, added to a normal incubate, did not impair the ordinary enzyme activity. A slight decrease in the activity of the control enzyme was observed also after the extensive dialysis which was reversed by GSH (Table XI).

Other SH-inhibitors. The effects of chloroacetophenone, iodoacetamide, N-ethyl-maleimide, phenylmercuric acetate, and o-iodosobenzoic acid were tested for their ability to inhibit the histamine methylation. All of them except o-iodosobenzoic acid had inhibitory effects. The inhibition was always prevented, and in some instances reversed by GSH. The results are compiled in Table XII.

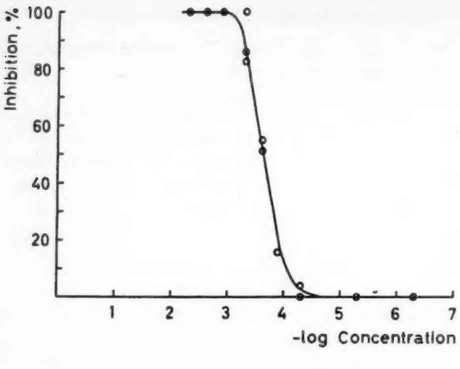


Fig. 6. *Inhibition of the methylhistamine formation by pCMB.*
Incubation under standard conditions for 30 min. Enzyme and inhibitor in the concentrations indicated were preincubated for 10 min.

Table IX. Histamine methylation in the presence of some presumptive methyl acceptors

The incubations were carried out at standard conditions for 30 min at 37° C. The histamine concentration is 0.17 mM.

Substance	Concentration, mM	Methylhistamine formation, per cent of control
Nicotinamide	2.5	92
	12.5	89
	15.0	97
Glycocyamine	0.75	103
	1.5	105
	3.75	100
	15.0	104
Homocysteine	1.25	102
	1.5	112
	15.0	116
Histidine	0.75	100
	1.5	114
	15.0	113
Cadaverine	1.5	96

Table X. The influence of adrenaline and noradrenaline on the histamine methylation

25 μl enzyme preparation was incubated for 30 min at 37° C under standard conditions, the histamine concentration being $1.7 \cdot 10^{-4}$ M. Methylhistamine was determined according to the chloroform method.

Substance	Concentration, M	Decrease in methylhistamine formation, %
Adrenaline	0.0015	9
	0.0075	32
	0.015	47
	0.075	65
	0.15	75
	[1] 0.075	46
Noradrenaline	0.0015	12
	0.0075	34
	0.015	43
	0.075	51
	0.15	74
	[1] 0.075	35

[1] 2.5 times the ordinary amount C^{14}-histamine was added.

(For a discussion of the properties of different SH-inhibitors see Barrow 1951 and Boyer 1959.)

Garlic extract. Allicin, a substance present in garlic extract, is a rather specific inhibitor of SH-enzymes (Wills 1956). Therefore garlic bulbs were crushed

Table XI. The effect of pCMB on the histamine methyl-transferase

Equivalent portions of enzyme were incubated with 0.034 μmole C^{14}-histamine and 0.1 μmole AMe in 0.5 M Tris buffer pH 8.0 to make 200 μl. Methylhistamine was determined by chloroform extraction after incubation for 30 min at 37° C.

	Per cent methylation
pCMB treated dialysed enzyme	20
untreated enzyme	56
both enzymes together	77
pCMB treated, dialysed enzyme + GSH 0.00125 M	61
dialysed enzyme	47
dialysed enzyme + GSH 0.00125 M	58

Table XII. The influence of some sulfhydryl reagents on histamine methylation

Incubation was performed under standard conditions for 30 min. The inhibitors were preincubated with the enzyme for 10 min before the substrate was added.

Substance	Concentration, M	GSH added before inhibitor, M	GSH added after the pre-incubation period, M	Methylation, % of control
Chloroacetophenone	$5 \cdot 10^{-5}$	—	—	100
	$5 \cdot 10^{-4}$	—	—	61
	$2.5 \cdot 10^{-3}$	—	—	19
	$5 \cdot 10^{-3}$	—	—	26
	$5 \cdot 10^{-5}$	$1.25 \cdot 10^{-4}$	—	100
	$2.5 \cdot 10^{-3}$	$2.5 \cdot 10^{-3}$	—	90
Iodoacetamide	$5 \cdot 10^{-4}$	—	—	88
	$5 \cdot 10^{-3}$	—	—	22
	$5 \cdot 10^{-3}$	$5 \cdot 10^{-3}$	—	89
	$5 \cdot 10^{-3}$	—	$5 \cdot 10^{-3}$	11
N-ethylmaleimide	$1 \cdot 10^{-4}$	—	—	73
	$1 \cdot 10^{-3}$	—	—	8
	$1 \cdot 10^{-3}$	$1 \cdot 10^{-3}$	—	100
	$1 \cdot 10^{-3}$	—	$1 \cdot 10^{-3}$	8
Phenylmercuriacetate	$2.5 \cdot 10^{-5}$	—	—	80
	$2.5 \cdot 10^{-4}$	—	—	6
	$2.5 \cdot 10^{-4}$	$1 \cdot 10^{-3}$	—	72
	$2.5 \cdot 10^{-4}$	—	$1 \cdot 10^{-3}$	105
pCMB	$5 \cdot 10^{-4}$	—	—	14
	$5 \cdot 10^{-4}$	$5 \cdot 10^{-4}$	—	89
	$5 \cdot 10^{-4}$	—	$1 \cdot 10^{-3}$	111
o-Iodosobenzoate	$2.5 \cdot 10^{-3}$			98
	$6.3 \cdot 10^{-3}$			103
	$1.25 \cdot 10^{-2}$			99
	$2.5 \cdot 10^{-2}$			96
	$1.25 \cdot 10^{-2}$	$1.25 \cdot 10^{-2}$		100

Table XIII. Inhibition of histamine methylation by garlic extract

The incubates contained 50 μl enzyme solution, 0.034 μmole C^{14}-histamine, 0.3 μmole AMe, and 0.5 M Tris buffer pH 8.0 to make 200 μl.

Garlic extract 1 : 5, μl	GSH 0.01 M, μl	Histamine methylated, %
—	—	90
5	—	46
10	—	8
—	25	88
5	10	86
10	10	83

and extracted with five times their own weight of water, and the juice was filtered. The histamine methylating enzyme was strongly inhibited by this extract (Table XIII). Addition of GSH protected the enzyme.

Aminoguanidine, iproniazid[7], *and IBINH*[8], powerful inhibitors of DO and monoamine oxidase, had no effect on the methylating enzyme. They were tested in concentrations up to $2 \cdot 10^{-4}$ M for aminoguanidine, and $2 \cdot 10^{-3}$ M for iproniazid and IBINH.

Relation between metals and enzymic activity

The influence of EDTA and of certain metal ions on the histamine methylation was tested. Only Cu^{2+} was found to have a pronounced effect (Table XIV).

Table XIV. The influence of metals on histamine methylation

Incubation was performed under standard conditions with the additions indicated. Methylhistamine formed within 30 min was determined with the chloroform method.

Additions	Histamine methylation, % of control	Additions	Histamine methylation, % of control
EDTA, 0.0025 M	109	$MnSO_4$, 0.001 M	84
EDTA, 0.025 M	100	$MnSO_4$, 0.01 M	63
$CaCl_2$, 0.001 M	93	Na_2SO_4, 0.001 M	100
$CaCl_2$, 0.01 M	95	Na_2SO_4, 0.01 M	95
$MgCl_2$, 0.001 M	96	$CuCl_2$, 0.001 M	11
$MgCl_2$, 0.01 M	93	$CuCl_2$, 0.01 M	4

[7] *Iproniazid* was generously supplied, free of charge, by Hoffman-La Roche, Basel.

[8] *IBINH* was kindly supplied by Drs. Lindell and Westling, Department of Physiology, Lund.

Table XV. Methylation of histamine in different mouse liver fractions

Samples of different cytoplasmic fractions, corresponding to equivalent amounts of liver were incubated for 60 min as follows: in exp. no. 1 and 2 the incubates contained 0.052 μmole C^{14}-histamine, 1.5 μmoles AMe, 0.1 g liver, and 0.5 M Tris pH 8.0 to a volume of 1 ml, in exp. no. 3 and 4 0.0069 μmole C^{14}-histamine, 0.25 μmole AMe, 0.006 g liver, and 115 μl 0.5 M Tris pH 8.0 in 200 μl.

Metabolites	Experiment no.	Homogenate	Mitochondrial fraction	Microsomal supernatant	Recombined fractions	High speed supernatant
Isotope dilution method:						
Histamine,	1	0	91	—	0	0
%	2	1	82	1	0	—
Methylhistamine,	1	96	1	—	96	99
%	2	70	1	98	79	—
MeImAA,	1	5	0	—	3	0
%	2	10	0	0	4	—
Chloroform extraction method:						
Methylhistamine,	3	95	0	95	—	—
%	4	77	0	91	80	84

Localization of the histamine methylating enzyme in liver cells

Mouse liver was homogenized in a Potter Elvehjem homogenizer in 0.25 M sucrose (Schneider and Hogeboom 1950). The homogenate (fraction 1) was centrifugated for 15 min at 600 *g*, and the precipitate discarded. The supernatant was fractionated by centrifuging successively at 5,000 *g* for 20 min (fraction 2) and 100,000 *g* for 60 min (fraction 3), the supernatants being saved. The precipitate obtained at 5,000 *g* was washed twice, and re-suspended (fraction 4). The four fractions thus obtained were arbitrarily designated as 1) homogenate, 2) microsomal supernatant, 3) high speed supernatant, and 4) mitochondrial fraction. The different fractions were incubated with C^{14}-histamine, each by itself, and recombined. Four experiments, each using liver from 6 mice were performed. In two experiments the formation of methylhistamine and MeImAA was determined with the isotope dilution method. In two experiments only methylhistamine was determined by the chloroform method (Table XV). As a criterion on the condition of the prepared mitochondria the O_2-uptake in the mitochondrial fraction with and without addition of hexokinase (Hunter Jr 1955) was simultaneously determined by Warburg technique.

In all experiments the formation of methylhistamine was localized to the supernatant fraction. No methylation was obtained in the mitochondrial

Table XVI. The occurrence of histamine methyl-transferase

0.5 ml enzyme extract was incubated with AMe and 0.125 μC C^{14}-histamine (corresponding to 0.026 or 0.084 μmole) in 0.5 M Tris buffer pH 7.4. After incubation at 37° C for 1 hr, radioactive histamine, methylhistamine, and MeImAA were determined with the isotope dilution method.

Species	% of radioactivity recovered as		
	Histamine	Methyl-histamine	MeImAA
Hamster	0.5	77	
Rat ♀	97	1	
Rat ♀	90	1	0.7
Rat ♂	67	1	0.6
Human ♀ (excl. AMe)	82		
Human ♀ (incl. AMe)	2		
Human ♀[1]	1	76	21
Human ♀	6	105	2
Human ♂	20	80	0.3
Rabbit[1]	5	49	22

[1] The liver homogenate was not centrifugated before use.

fraction alone. Addition of mitochondrial fraction to the supernatant did not significantly alter the methylation. In the homogenate and the recombined mitochondrial and supernatant fractions a further metabolization of methylhistamine may possibly have occurred in contrast to the case of the supernatant fraction alone, where only methylation takes place.

On addition of hexokinase the respiration of the mitochondrial fraction increased about four times.

Occurrence of the histamine methyltransferase

Liver from different animals was tested on its ability to methylate histamine. Enzyme extracts were prepared simply by grinding the tissue in three times its own weight of sodium acetate buffer pH 5.6, and centrifugating briefly. The supernatant was used for the experiments directly or after dialysis against NaAc buffer. The histamine metabolites were determined with the isotope dilution method. The results are shown in Table XVI. Of the livers tested only rat liver was incapable of forming methylhistamine.

Three rabbit livers (from two males, one female) were tested with the chloroform method. They all were able to synthesize methylhistamine. Also cat liver extract was incubated with C^{14}-histamine. The entire radioactivity was extractable into chloroform after 30 min. incubation of 50 μl enzyme with 1.27 μg C^{14}-histamine.

Aminoguanidine and iproniazid (or IBINH) had little or no effect on the disappearance of histamine in the liver extracts tested (Table XVII).

Table XVII. The influence of aminoguanidine and iproniazid on histamine methylation by liver extracts

0.5 ml liver extract was incubated at 37° C for 1 hr with 1.5 μmoles AMe, 0.125 μC (0.026—0.084 μmole) C^{14}-histamine, and 0.5 M Tris buffer pH 7.4 to 1 ml. The remaining histamine was determined by the isotope dilution method.

Species	Histamine recovered, %		
	No inhibitor	Aminoguanidine 10^{-4} M	Iproniazid, 10^{-3} M
Mouse	0.6	18	[1] 0.5
Pig	0.9	0.8	
Rat	97	96	
Human ♀	2	3	3
Human ♂	20	21	19
Rabbit	5	7	

[1] In this experiment, IBINH is substituted for iproniazid.

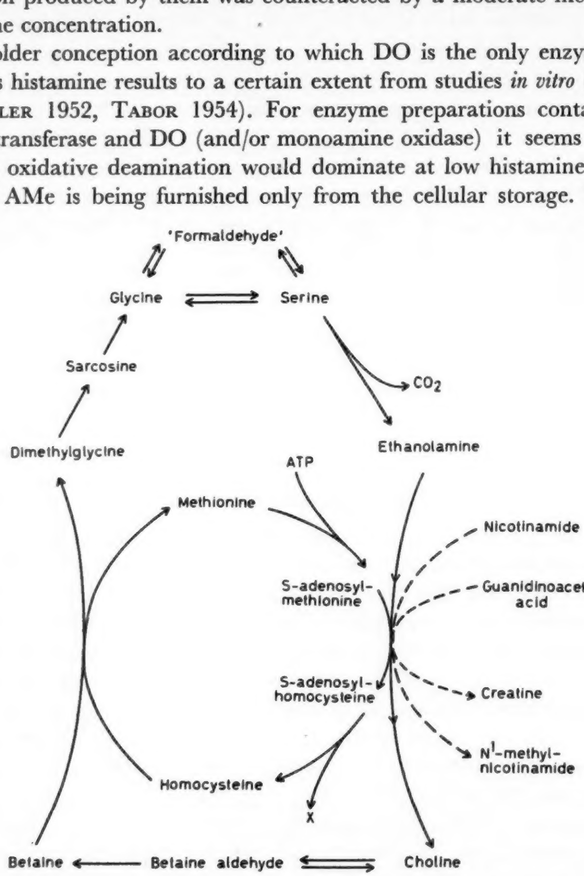
Discussion

During the last decade a new conception of the synthesis and transfer of methyl groups has developed. An instructive diagram was given by Ericson *et al.* in 1955 (Ericson, Williams and Elvehjem 1955, see page 134).

It is now possible to include the methylation of noradrenaline to adrenaline (Kirshner and Goodall 1957) as well as the O-methylation of both amines (Axelrod 1957, Axelrod, Senoh, and Witkop 1958, Axelrod and Tomchick 1958) in this scheme in the same manner as shown for glycocyamine (guanidinoacetic acid), and nicotinamide. It has been shown in the present paper that also histamine belongs to the same group of methyl acceptors: betaine or methionine can not replace AMe as methyl donors in the presence of partly purified histamine methyl-transferase. Thus it appears that AMe functions as a methyl dispenser for the transformation of both catechol amines and histamine. However, the specificity studies do not imply that the different methylation reactions using AMe as methyl donor should be catalyzed by the same enzyme. Nicotinamide and glycocyamine certainly do not influence the enzyme studied here. This does not mean that these substances must be without effect *in vivo*. It may well be that in massive doses glycocyamine or nicotinamide are able to impair the histamine methylation *via* a depletion of available AMe. Recently Udenfriend *et al.* has shown that the methylation *in vivo* of noradrenaline is inhibited by the substances in question (Udenfriend, Creveling, Ozaki, Daly and Witkop 1959). The slight inhibition of the histamine methylation found to be induced by high concentrations of adrenaline and noradrenaline would seem to be rather unspecific. Anyhow the affinity of the enzyme to

histamine seems to be much greater than to the two former substances, since the inhibition produced by them was counteracted by a moderate increase in the histamine concentration.

The older conception according to which DO is the only enzyme that inactivates histamine results to a certain extent from studies *in vitro* (for reviews see ZELLER 1952, TABOR 1954). For enzyme preparations containing both methyl-transferase and DO (and/or monoamine oxidase) it seems likely also that the oxidative deamination would dominate at low histamine concentrations, if AMe is being furnished only from the cellular storage. This might



explain, why the methylating enzyme has escaped observation. In undamaged cells the conditions might be quite different. It would seem as if the two enzymes were localized in different parts of the cell. The intracellular localization of DO has been studied by COTZIAS and DOLE (1952). Using rabbit liver they found that the enzyme is predominantly associated with the particulate components of the cell. The mouse liver histaminase discovered by KOBAYASHI (1957) seems to be located in the mitochondria. Our experiments suggest that histamine methyl-transferase is a component of the soluble cytoplasm. However, too much weight must not be attributed to the results obtained: the determinations performed indicate that the mitochondria in our preparations

were undamaged from the point of view of common practice, but it is nevertheless by no means definitely proved that nothing has leaked out from subcellular particles. (For a discussion of the problems involved in localization experiments see SCHNEIDER 1959.)

In respect to inhibitors there exists a marked difference between DO and histamine methyl-transferase. Aminoguanidine is without any effect on the methylation. Neither do iproniazid and IBINH produce any effect on the methylation step, but they block the subsequent oxidation. SH-inhibitors on the other hand are reported to be without inhibitory effect on DO (for ref. see BONETTI and BOMBARA 1957), but are able completely to inhibit the methylation.

It is very difficult indeed to prove, by use of inhibitors, that an enzyme has essential SH-groups (BARROW 1951, BOYER 1959). However, the experiments reported here suggest very strongly that histamine methyl-transferase has essential SH-groups: o-iodosobenzoate admittedly had no effect, but it is well known that by reason of essentially steric effects not all of the SH-groups in a protein may be available for oxidation[*]. The alkylating and mercaptide forming agents were all inhibitory. Of the inhibitors used pCMB is usually considered to be the most specific, especially when accompanied as here by reactivation. The effect of pCMB is not due to the replacement of non-polar SH-groups by negatively charged groups, as phenyl mercuric acetate is even more effective. Evidently pCMB exerts its effect on the enzyme itself, and not only on the substrates, since pre-incubation of the enzyme and the inhibitor with subsequent dialysis leaves an inactive enzyme.

Also the shape of the pH curve might have a bearing on the postulated SH-dependence. Values for the dissociation of protein-SH groups can admittedly only be approximated by analogy with simpler compounds. It might, however, deserve notice that BENESCH and BENESCH (1955) obtained apparent pK values 8.53 and 10.03 for the thiol group in cysteine ($HSRNH_3^+ \rightleftharpoons {}^-SRNH_3^+$ and $HSRNH_2 \rightleftharpoons {}^-SRNH_2$, respectively). Anyhow the dissociation constants are bound to be rather high as exemplified by the observation that only 1 % of *e. g.* GSH and DL-homocysteine appear in RS^--form at pH 7.4 (BENESCH 1955). The sudden inactivation of the enzyme at pH over 10 points to denaturation of the enzyme protein: pre-incubation at pH 10.8 results in irreversible loss of enzyme activity.

The shape of the pH curve is evidently open to other interpretations. The ionization constants of histamine are about 6.03 and 9.86 (ALBERT 1952). Thus below pH 6 histamine exists mainly in dicationic form. It would seem as if the dicationic form of histamine is a poorer substrate of histamine methyl-

[*] Addendum: In later experiments performed with an extensively purified enzyme preparation the enzyme activity was inhibited by o-iodosobenzoate. The inhibition was reversed by addition of GSH.

Possibly the inhibition is counteracted by inpurities in the experiments described above.

transferase than the monocation and the non-polar form. This again must be seen against the background that DO has an optimum at pH 6 (Westling and Nilsson 1959), whereas the mouse liver histaminase discovered by Kobayashi (1957) and the plasma oxidase described by Blaschko (Blaschko, Friedman, Hawes and Nilsson 1959) have optima above pH 8.

The few results on the occurrence of histamine methyl-transferase accounted for here are all in accordance with the findings *in vivo* by Schayer (1956). He found that homo, cat, mouse, and rabbit, but not the rat to any appreciable extent, excreted methylated histamine derivatives. Later Westling (1958) has found significant quantities of methylhistamine in rat urine. He reports a sex difference in the histamine metabolism of the rat. Provided that histamine methyl-transferase occurs also in the rat, there must be other points *e. g.* with regard to the localization or the further metabolization, which differ from those found in the other species studied.

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Gesamthämoglobinmenge und Blutvolumen der normalen, anämischen und polycythämischen Ratte, bestimmt mit CO und Cr^{51}

Von

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Abstract

ENGSTEDT, L., B. PERIĆ und B. TRIBUKAIT. *Gesamthämoglobinmenge und Blutvolumen der normalen, anämischen und polycythämischen Ratte, bestimmt mit CO und Cr^{51}*. Acta physiol. scand. 1960. *49*. 139—147. — In normal, anemic and polycythemic rats the blood volume and the total amount of hemoglobin have been determined by means of a modified alveolar CO method and Cr^{51} tagged erythrocytes. The Cr^{51} method gives on an average 10 per cent lower values than the CO method, both in normal, anemic and polycythemic animals. Reasons for this difference are discussed.

Das Blutvolumen der Ratte wird in der Literatur mit 4.59 bis 7.98 ml/100 g Körpergewicht angegeben (BERLIN *et al.* 1949, BECKWITH und CHANUTIN 1941). Eine wesentliche Ursache für diese Differenzen ist neben Unterschieden der Tierstämme, des Alters und der äusseren Lebensbedingungen in Eigenheiten der verwendeten Methoden zu suchen. Da der Körperhämatocrit unter dem des zentralen Gefässystems liegt — ihr Verhältnis beträgt bei der Ratte nach EVERETT, SIMMONS und LASHER (1956) 0.88, nach HUANG und BONDURANT (1956) 0.98 und nach WANG (1959) 0.74 — ergibt das aus dem Plasmavolumen (Evans Blue, Congorot, J^{131}) und dem zentralen Hämatocrit bestimmte Blutvolumen zu grosse, das aus dem Zellvolumen (Cr^{51}, Fe^{59}, P^{32}) und dem zentralen Hämatocrit bestimmte Blutvolumen zu kleine Werte.

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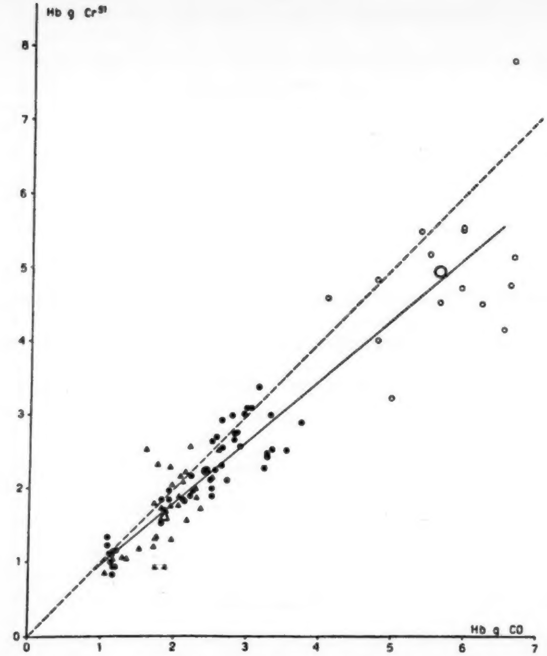

Abb. 1. Vergleich der mit Cr^{51} (Ordinate) und CO (Abszisse) bestimmten Hämoglobinwerte. Gleichung der Regressionslinie: $y = 0.83\ x + 0.15$; $r = 0.93$. Die ausgezogene Regressionslinie unterscheidet sich hochsignifikant von der 45°-Linie.
● = Normaltiere △ = Anämietiere ○ = Polycythämietiere.
Die grossen Symbole bezeichnen die Mittelwerte der jeweiligen Gruppen.

In der vorliegenden Arbeit sind Hämoglobinmenge und Blutvolumen derselben Ratten mit einer zur wiederholten Bestimmung weiterentwickelten alveolaren CO-Methode und mit einer Zellverdünnungsmethode (Cr^{51}) gemessen und verglichen worden. Die Untersuchungen erstrecken sich auf normale Ratten unterschiedlicher Grösse, anämische und polycythämische Tiere.

Methodik

Die Hämoglobinmenge und das Blutvolumen von 150—550 g schweren männlichen Tieren eines Stammes (Wistar) wurden gleichzeitig mit CO (Tribukait 1960a) und Cr^{51}-gezeichneten Erythrocyten gemessen. Dafür wurde heparinisiertes Blut von Spendertieren zentrifugiert, das Plasma abpipettiert und Cr^{51} als Na-Chromat mit einer spezifischen Aktivität von etwa 10 μc/ml Erythrocyten zugesetzt. Nach 30 Minuten Inkubationszeit bei Zimmertemperatur wurden die Erythrocyten 2 mal mit dem etwa 5fachen Volumen isotonischer Kochsalzlösung gewaschen, wodurch praktisch alle freie Radioaktivität entfernt wird.

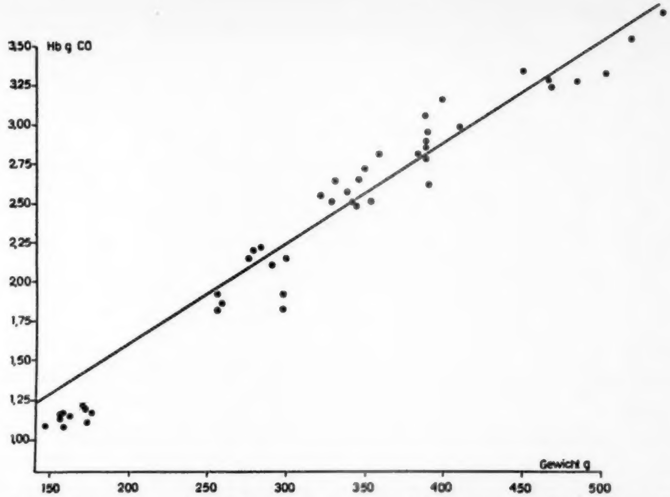

Abb. 2. Beziehung der mit CO bestimmten Hämoglobinmenge (Ordinate) zum Körpergewicht (Abszisse). Gleichung der Regressionslinie: $y = 0.0064\,x + 0.33$; $r = 0.89$.

Von dieser Erythrocytensuspension wurde den Tieren, denen in Äthernarkose die V. cava inf. freigelegt worden war, mit einer standardisierten Spritze 0.239 ± 0.00011 ml injiziert. Die injizierte Aktivität wurde dadurch bestimmt, dass die gleiche Menge Blut in Saponinlösung hämolysiert und in einem Scintillationszähler gemessen wurde. Vor Injektion der Erythrocyten wurde die Hämoglobinkonzentration vom Blut der coupierten Schwanzspitze festgestellt (Methodik siehe Tribukait 1960a), der nach einer Durchmischungszeit der injizierten Erythrocyten von 10 Minuten mit einer eingewogenen Mikropipette 0.1 ml Blut zur Bestimmung der Aktivität entnommen wurde und bei Doppelbestimmungen gleichzeitig eine weitere Probe für die relative Hämoglobinkonzentration. Die gefundene Aktivität wurde dann zur Ausgangsaktivität.

Die vom Körperhämatocrit unabhängige Gesamthämoglobinmenge (Tot. Hb in g) ergibt sich aus der injizierten Aktivität (a), der gefundenen Aktivität (b) und der Hämoglobinkonzentration (rel. Hb in g %) vor der Injektion entsprechend der Gleichung

$$\text{Tot.Hb} = \frac{a \times \text{rel. Hb}}{b},$$

das Blutvolumen (B. V. in ml) aus der Gleichung

$$\text{B.V.} = \frac{a}{b \times 0.75}.$$

0.75 ist ein Korrekturfaktor für den Körperhämatocrit (Tribukait 1960b). Das injizierte Volumen von 0.239 ml ist so gering, dass eine Korrektur dafür bei der Berechnung der Gesamthämoglobinmenge unnötig erschien.

Die Anämie wurde nach Abschluss einer Cr^{51}-Bestimmung durch eine einmalige Blutentnahme erzeugt, auf welche 2 Tage später eine erneute Bestimmung folgte. Die Restaktivität wurde zuvor in 0.1 ml Schwanzblut bestimmt. Die Polycythämie wurde durch mehrwöchigen Unterdruck entsprechend 6,000 m Höhe hervorgerufen (Einzelheiten dazu siehe Tribukait 1960d,e).

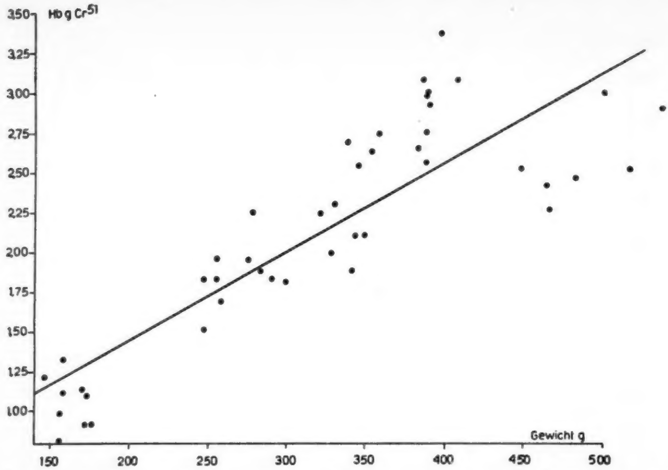

Abb. 3. Beziehung der mit Cr^{51} bestimmten Hämoglobinmenge (Ordinate) zum Körpergewicht (Abszisse). Gleichung der Regressionslinie: $y = 0.0055\,x + 0.35$; $r = 0.86$.

Resultate

Einen Gesamtüberblick über die gleichzeitig mit Cr^{51} (Ordinate) und CO (Abszisse) gefundenen Hämoglobinmengen vermittelt Abb. 1. Die Tendenz, mit CO höhere Werte als mit Cr^{51} zu bekommen, ist ganz offenbar. Dabei verhalten sich die verschiedenen Tierarten recht gleichmässig. Der Quotient Cr^{51}-/CO-gemessener Werte von 45 Normaltieren ist 0.911 ± 0.019, von 32 Anämietieren 0.891 ± 0.036 und von 15 Polycythämietieren 0.877 ± 0.043. Zwischen diesen Werten besteht keine statistisch zu sichernde Differenz. Neben diesem systematischen Unterschied sind Schwankungen der Einzelwerte deutlich.

Zur weiteren Analyse sind mit beiden Methoden Fehleruntersuchungen an Hand von Doppelbestimmungen durchgeführt worden, mit CO an 2 aufeinander folgenden Tagen, mit Cr^{51} im Anschluss an die erste Messung. In 35 Versuchen betrug für CO der Variationskoeffizient 6.2 % und für Cr^{51} 10.4 %. Da zwischen den Werten des ersten und zweiten Cr^{51}-Versuchs kein statistisch zu sichernder Unterschied besteht (p 0.4—0.3), scheint die Durchmischung nach 10 Minuten vollständig zu sein. 13 Doppelbestimmungen des Standards (= injizierte Aktivität) ergaben einen Variationskoeffizienten von 3.5 %.

Es darf angenommen werden, dass verschiedene Tiere gleichen Stammes, gleichen Gewichts und unter gleichen Lebensbedingungen auch eine etwa gleichgrosse Hämoglobinmenge haben. Die Richtigkeit dieser Annahme lässt sich gut aus Abb. 2 erkennen, in der die mit CO gemessenen Werte auf der

Tabelle I. Verhältnis der mit Cr^{51}-/CO-gemessenen Werte in verschiedenen Einzelgruppen von Tieren vor und 2 Tage nach Blutentnahme

Gruppe Nr.	Zahl	Cr^{51}/CO		Signifikanz der Differenz
		vor	nach	
I	7	0.76 ± 0.025	0.87 ± 0.058	$p > 0.05$
II	9	1.02 ± 0.028	1.00 ± 0.067	$p > 0.05$
III	10	0.89 ± 0.035	0.84 ± 0.085	$p > 0.05$
IV	6	0.92 ± 0.022	0.83 ± 0.029	p 0.05—0.01

Ordinate gegen das Gewicht auf der Abszisse aufgetragen sind. Behandelt man in gleicher Weise die mit Cr^{51}-gemessenen Werte (Abb. 3), lassen sich erheblich grössere Schwankungen, vor allen Dingen bei den schwereren Tieren, feststellen.

Das wird aus Tab. I deutlich, in der der Quotient der mit Cr^{51}- und CO-bestimmten Werte für verschiedene Tiergruppen vor und 2 Tage nach einer Blutentnahme gebildet ist. Tab. I zeigt weiter, dass sich dieser Quotient während akuter Anämie praktisch nicht verändert.

Über das Verhalten des Blutvolumens vor und während Anämie geben Tab. II und Abb. 4 a und 4 b Aufschluss. Dort sind teilweise die für die verschiedenen Tiergruppen mit beiden Methoden gefundenen Mittelwerte zusammengefasst, teilweise alle erhaltenen Einzelwerte aufgezeichnet. Geht man von den mit CO bestimmten Werten aus, ist das Blutvolumen der grössten Tiere (Gruppe I), obwohl 7 ml abgenommen worden sind, unverändert. Ein Blutverlust von 7 ml bei etwas kleineren Tieren (Gruppe II) führt zu einem hochsignifikant geringeren Blutvolumen, ebenfalls bei den noch kleineren Tieren der Gruppe III, denen nur 5 ml abgenommen worden sind. Bei den kleinsten Tieren (Gruppe IV) ist mit CO der Unterschied nicht statistisch signifikant, für Cr^{51} schwach signifikant. In den Gruppen I—III der Cr^{51}-Werte sind die Variationen so gross, wie auch leicht aus Abb. 4 a zu erkennen, dass kein statistisch zu sichernder Unterschied nachweisbar ist.

Diskussion

Vergleichende Untersuchungen des Blutvolumens, berechnet aus dem mit radioaktiv gezeichneten Erythrocyten bestimmten Erythrocytenvolumen und der mit CO gemessenen Hämoglobinmenge, haben für das mit CO bestimmte Blutvolumen stets höhere Werte ergeben (Hevesy *et al.* 1944 — homo, Root, Allen und Gregersen, 1953 — Hund, Nomof *et al.* 1954 — homo, Wennesland *et al.* 1957 — Kaninchen). Für diese Differenzen, die bis zu 40 %

Tabelle II. Blutvolumen verschiedener Einzelgruppen von Tieren, bestimmt mit Cr^{51} und CO, vor und 2 Tage nach Blutentnahme

Gruppe Nr.	Zahl	abgen. Blutmenge ml	Blutvol. Cr^{51}		Signif. d. Diff.	Blutvol. CO		Signif. d. Diff.
			vor	nach		vor	nach	
I	7	7.0	23.3 ± 1.44	24.7 ± 1.72	$p > 0.05$	29.1 ± 0.74	28.2 ± 0.52	$p > 0.05$
II	9	7.0	23.9 ± 0.71	21.9 ± 1.62	$p > 0.05$	24.1 ± 0.56	20.4 ± 0.56	$p < 0.001$
III	10	5.0	18.9 ± 0.54	17.4 ± 1.49	$p > 0.05$	21.3 ± 0.36	19.1 ± 0.26	$p < 0.001$
IV	6	5.0	14.6 ± 0.39	13.1 ± 0.45	p0.05—0.01	17.2 ± 0.59	15.7 ± 0.69	$p > 0.05$

ausmachen, werden von den Autoren nicht Ungenauigkeiten bei der Anwendung der Methoden, sondern eine Bindung von CO an nicht zirkulierendes Hämoglobin und Myoglobin vermutet. SJÖSTRAND (1948) nimmt für die von ihm entwickelte alveolare CO-Methode einen 5 %-igen CO-Verlust durch Adsorption an Myoglobin an. Die mit dieser Methode und einer Zellverdünnungsmethode (P^{32}) erhaltenen Werte für das Gesamthämoglobin waren übereinstimmend (WICKLANDER 1956).

In den vorliegenden Versuchen sind die mit Cr^{51} gemessenen Werte durchschnittlich um etwa 10 % niedriger als die mit CO bestimmten. Es liegt nahe, die Differenz ebenfalls auf eine Bindung von CO and Myoglobin und an im Knochenmark fixiertes Hämoglobin zurückzuführen. Für die Ratte mit etwa 0.1 g Myoglobin und 0.08 g im Knochenmark liegendem Hämoglobin ist jedoch der Anteil der von diesen Substanzen gebundenen CO-Menge nur auf maximal 5 % der insgesamt aufgenommenen CO-Menge kalkuliert worden (TRIBUKAIT 1960a). Die Frage, ob dieser Wert zu niedrig ist, können einige unserer Daten näher beleuchten.

Bei einem Anstieg der Gesamthämoglobinmenge um 150 bis 200 % in Hypoxie ist auch die Cr^{51}-CO-Differenz entsprechend angestiegen. Das ist deshalb erstaunlich, weil offenbar das Knochenmarkvolumen in Hypoxie unverändert ist (HUDSON 1958), die Hämoglobinkonzentration des hypoxischen Knochenmarks ebenfalls nicht ansteigt (TRIBUKAIT 1959 unveröffentlicht) und auch die Myoglobinmenge in Hypoxie unverändert scheint (ANTHONY, ACKERMANN und STROTHER 1959). Auch bei den anämischen Tieren hat sich die relative Cr^{51}-CO-Differenz im ganzen nicht verändert. Diese sollte jedoch grösser werden, wenn sie auf einer CO-Bindung an Myoglobin und nicht zirkulierendes Hämoglobin beruht.

Unerwartet gibt es einzelne Tiergruppen, deren CO- und Cr^{51}-Werte keine Differenzen aufweisen, die anderer dagegen umso grössere. Im Hinblick darauf, dass die Relation Hämoglobinmenge/Körpergewicht für CO gut, für

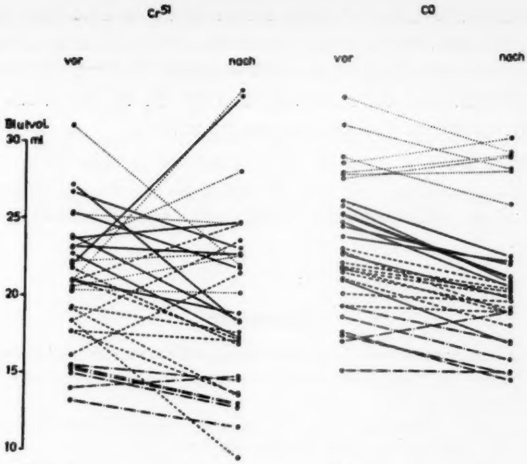

Abb. 4 a. 4 b.

Abb. 4 a und b. Einzelwerte der Blutvolumina, bestimmt mit Cr^{51} und CO, vor und 2 Tage nach einer Blutentnahme. Mit den unterschiedlichen Linien werden die Tiere verschiedener Tiergruppen gekennzeichnet.

Cr^{51} schlecht ist, erscheint es berechtigt, die Ursache dafür eher bei der Cr^{51}-Methode zu suchen. Fixation von radioaktivem Cr^{51} im Gewebe, eventuell im Zusammenhang mit Hämolyse, würde eine zu grosse Hämoglobinmenge vortäuschen; mangelnde Durchmischung des peripheren Blutes, wie sie bei einer Agglutination der radioaktiv gezeichneten Erythrocyten oder im Schock denkbar wäre, eine zu kleine. Dass Cr^{51} von Lunge und Milz aufgenommen werden kann, ist bekannt (Wennesland *et al.* 1957). In unseren Versuchen ist eine Hämolyse des zu injizierenden Blutes stets sorgfältig vermieden worden und auch eine einsetzende Hämolyse nach Injektion erscheint unwahrscheinlich, da bei Doppelbestimmungen der zweite Wert gegenüber dem ersten nicht grösser zu sein pflegte. Möglicherweise sprechen aber Versuche von Pareira, Serkes und Lang (1959), die das Erythrocytenvolumen von Ratten im Schock mit radioaktiv gezeichneten Erythrocyten bestimmten und weder für Durchmischungszeit noch Erythrocytenvolumen eine Differenz zu den Kontrollen fanden, dafür, dass sich die dem Organismus fremden radioaktiv gezeichneten Erythrocyten normalerweise nicht vollständig mit dem Kapillarblut mischen.

Der 10 %-ige Methodenfehler mit Cr^{51} ist teilweise auf die Ungenauigkeit der injizierten Aktivität mit einem Variationskoeffizienten von 3.5 % zurückzuführen. Da das Flüssigkeitsvolumen, das mit der standardisierten Injektionsspritze gemessen wird, eine erheblich grössere Genauigkeit hat, ist dieser Fehler überwiegend Folge einer inhomogenen Aktivitätsverteilung der Erythrocytenaufschwemmung.

Das Blutvolumen bei Anämie zeigt im ganzen eine Tendenz zu niedrigeren Werten als in früheren Versuchen (TRIBUKAIT 1960c). Das mag darauf zurückzuführen sein, dass die abgenommene Blutmenge hier im allgemeinen grösser war und möglicherweise auch darauf, dass die Tiere durch die Bestimmung mit Cr^{51} einem stärkeren Trauma ausgesetzt waren.

Die Untersuchungen wurden aus Prof. T. SJÖSTRAND zur Verfügung gestellten Mitteln »Stiftelsen Therese och Johan Anderssons Minne« und »Riksidrottsförbundets Poliklinikkommitté« bestritten.

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Verhalten von Gesamthämoglobin und Blutvolumen der Ratte bei akuter Blutungsanämie

Von

BERNHARD TRIBUKAIT

Eingegangen am 17. Dezember 1959

Abstract

TRIBUKAIT, B. *Verhalten von Gesamthämoglobin und Blutvolumen der Ratte bei akuter Blutungsanämie.* Acta physiol. scand. 1960. *49.* 148—154. — After withdrawal, by venesection, of about 30 % of the blood volume of a rat the total amount of hemoglobin and blood volume was repeatedly estimated by a modified alveolar CO method. At about 3 days following the venesection the amount of hemoglobin started to increase. On the 20th day the initial amount of hemoglobin was regained. The daily production of hemoglobin reaches a maximum 3—4 days after venesection and returns to normal values within about 3 weeks. The blood volume, estimated from 24 hours to 15 days after the venesection was unchanged and as large as before the venesection.

Unsere Kenntnisse über den Regenerationsverlauf der Gesamthämoglobinmenge nach akuter Blutung gründen sich vorwiegend auf Veränderungen der Hämoglobinkonzentration des Blutes. Wegen der Schwankungen des Plasmavolumens, vor allem in der ersten Zeit nach der Blutung, sind die auf diese Weise gewonnenen Daten über die Veränderung der Hämoglobinmenge unsicher.

Die Auffassungen über das Verhalten des Blutvolumens nach akuter Blutung sind nicht einheitlich, obwohl in verschiedenen Untersuchungen das Gesamtblutvolumen vor und nach einer Blutung bestimmt worden ist. EBERT, STEAD und GIBSON (1941) fanden beim Menschen 3—4 Tage, COURTICE und GUNTON (1949) beim Kaninchen bereits 2 Stunden nach der Blutung ein normal grosses Blutvolumen, BOCK (1921), BENNETT *et al.* (1938) beim Menschen

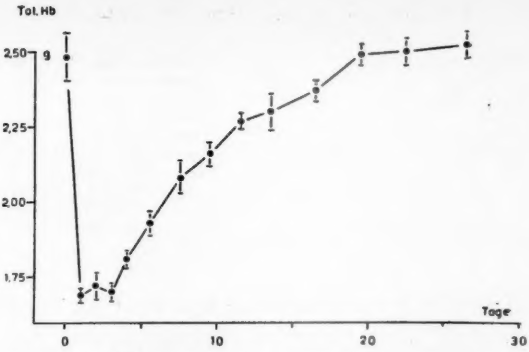

Abb. 1. Regeneration des Gesamthämoglobins nach einer Blutentnahme. Ordinatenachse: Gesamthämoglobin in g; Abszissenachse: Zeit in Tagen. Mittelwerte von 44 Tieren; die vertikalen Linien bezeichnen die mittleren Fehler der Mittelwerte.

sowie SMITH und SIMMONDS (1954) beim Kaninchen ein geringeres. Art der verwendeten Bestimmungsmethode, Grösse der Blutung, Zeitpunkt der Bestimmung nach der Blutung und der Hydrationszustand des Versuchsobjektes kommen vorwiegend als Ursache der Differenzen in Frage.

Eine weiterentwickelte sogenannte alveolare CO-Methode ermöglicht es, wiederholt die Hämoglobinmenge und das Blutvolumen der Ratte zu bestimmen. Damit soll nachfolgend zu erklären versucht werden, 1. wann nach einer akuten Blutung die Hämoglobinmenge anzusteigen beginnt, 2. mit welcher Geschwindigkeit die Regeneration vor sicht geht und nach welcher Zeit der Ausgangswert erreicht wird, 3. wie sich das Blutvolumen in den verschiedenen Regenerationsphasen verhält.

Methodik

250—350 g schweren männlichen Ratten eines Stammes (Wistar) wurde in Äthernarkose unter peinlicher Vermeidung von Blutungen die V. cava inf. freigelegt, der etwa 1/3 der Hämoglobinmenge bzw. 25—30 % des Blutvolumens mit einer Spritze entnommen wurde.

Die Gesamthämoglobinmenge vor und an verschiedenen Tagen nach der Blutentnahme wurde mit der von TRIBUKAIT (1959 a) beschriebenen Methode bestimmt, das Blutvolumen aus dem Gesamthämoglobin und der Hämoglobinkonzentration des Schwanzblutes × 0.75 berechnet. 0.75 ist ein Umrechnungsfaktor für den Körperhämatocrit (TRIBUKAIT 1960 b).

Ergebnisse

Der Regenerationsverlauf der Hämoglobinmenge von 44 Ratten ist in Abb. 1 dargestellt. Nach Blutentnahme am Tage 0 ist die Hämoglobinmenge von einem Mittelwert von 2.48 g auf 1.69 g abgesunken. Am 3. Tag nach der Blutentnahme setzt offenbar die Regeneration der Hämoglobinmenge ein,

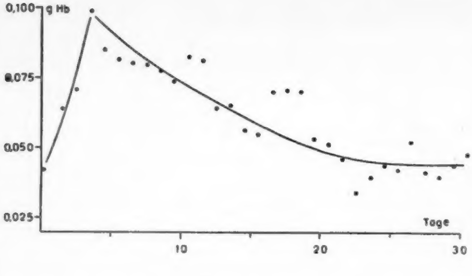

Abb. 2. Hämoglobinbildung/Tag nach einer Blutentnahme. Ordinatenachse: Hämoglobin in g/Tag; Abszissenachse: Zeit in Tagen, Mittelwerte von 44 Tieren.

die am 4. Tag mit 1.81 g signifikant über dem Wert des Vortages liegt. Am 10. Tag sind rund 60 % der verlorenen Hämoglobinmenge neu gebildet, am 20. Tag ist das Ausgangsniveau erreicht.

Die pro Tag gebildete Totalhämoglobinmenge ist teils aus der Abbaurate, die durch die Erythrocytenlebenszeit bedingt ist, teils aus den Differenzen der zu verschiedenen Zeitpunkten bestimmten Gesamthämoglobinmenge berechnet worden. Für die Lebenszeit der Erythrocyten von Ratten sind 60 Tage angenommen worden (Berlin, Meyer und Lazarus 1951, Fryers und Berlin 1952, Berlin, van Dyke und Lotz 1953, Smith, Odell und Caldwell 1959).

Die Ergebnisse dieser Berechnungen, denen Daten von 44 Tieren zugrunde liegen, sind in Abb. 2 zusammengefasst. Die mittlere Hämoglobinbildung/Tag steigt gegenüber einem Normalwert von 0.0418 g zwischen dem 3. und 4. Tag nach der Blutung auf 0.0990 g zu einem Maximum an, fällt dann mit erheblichen Schwankungen langsam ab und hat etwa am 23. Tag das Ausgangsniveau erreicht.

Über das Blutvolumen während Anämie gibt Tab. I Aufschluss. Da in diesen Versuchen nicht von allen Tieren zu den verschiedenen Zeitpunkten nach der Entblutung gleichzeitig das Blutvolumen bestimmt worden ist, sind

Tab. I. Blutvolumen von Ratten nach Blutentnahme. Den zu verschiedenen Zeiten gemessenen Werten sind die entsprechenden Ausgangswerte gegenübergestellt

Tage nach Entblutung	Zahl	Blutvolumen in ml	
		vor	nach Blutung
1	44	22.8 ± 0.40	22.5 ± 0.49
2	20	24.3 ± 0.49	25.1 ± 0.69
3— 4	20	24.0 ± 0.82	25.1 ± 0.68
5— 6	12	23.3 ± 0.84	24.0 ± 0.90
8	11	21.2 ± 0.74	22.8 ± 0.78
12—15	16	23.0 ± 0.71	23.5 ± 0.67

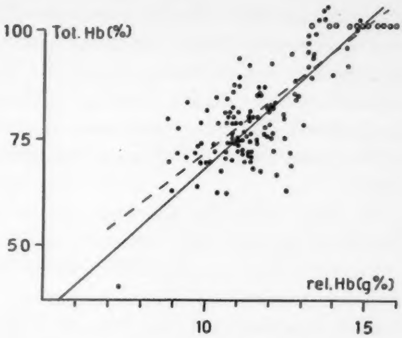

Abb. 3. Verhalten der Hämoglobinkonzentration des Blutes bei Verminderung der Gesamthämoglobinmenge. Ordinatenachse: Gesamthämoglobin in %, Ausgangswert = 100 %; Abszissenachse: Hämoglobinkonzentration in g/100 ml Blut. Jeweils 5 der Ausgangswerte sind in einem Punkt (offene Symbole) zusammengefasst. Ausgezogene Linie: Verbindung zwischen 0-Punkt und Mittelwert der Ausgangslinie. Gleichung der Regressionslinie (gestrichelte Linie): $y = 5.72x + 13.74$; $r = 0.86$.

die gefundenen Werte jeweils mit den entsprechenden Ausgangswerten verglichen worden. Zu keinem Zeitpunkt, auch nicht am 1. Tag nach der Blutung, hat sich das Blutvolumen gegenüber dem Ausgangswert statistisch nachweisbar verändert. Das bedeutet, dass der Blutverlust jeweils voll durch einen Anstieg des Plasmavolumens kompensiert worden ist, und dass die Hämoglobinkonzentration entsprechend dem Totalhämoglobinverlust abgesunken ist.

Das geht auch aus Abb. 3 hervor, in der die Gesamthämoglobinmenge in % auf der Ordinate gegen die unkorrigierte Hämoglobinkonzentration des Schwanzblutes auf der Abszisse aufgetragen worden ist. Die Werte verteilen sich um die ausgezogene Verbindungslinie zwischen 0-Punkt und dem Mittel der Ausgangswerte ziemlich regelmässig. Zwischen dieser Verbindungslinie und der gestrichelten Regressionslinie besteht keine statistisch zu sichernde Differenz.

Diskussion

Anämie ist neben Hypoxie einer der stärksten Reize der Erythropoiese. Rund 72 Stunden nach Beginn des anämischen Reizes setzte in den vorliegenden Versuchen die Hämoglobinregeneration ein. Auf Grund von Untersuchungen mit indirekten Methoden teilweise auch bei grösseren Tieren, bei denen ein Operationstrauma vermieden wurde, und beim Menschen, darf als wahrscheinlich angenommen werden, dass diese Latenz weniger Folge des mit der Blutentnahme verbundenen Operationstraumas als vielmehr Ausdruck der notwendigen Bildungszeit der Erythrocyten und der Hämoglobinsynthese ist. Whipple und Robscheit-Robbins (1936) sahen bei Hämoglobinumsatzbestimmungen am anämischen Hund gewöhnlich am 3. Tag nach Zusatz eines für die Blutbildung wirksamen Diätfaktors den Anstieg der Hämoglobinproduktion. Mit Hilfe autoradiographischer Untersuchungen des Knochenmarks der mit radioaktivem Eisen vorbehandelten Ratte haben Harriss (1957) für die Entwicklungszeit des frühen Normoblasten bis zum

polychromatischen Erythrocyten des Blutes etwa 2 Tage angesetzt, ALPEN und CRANMORE (1959) mit derselben Methode beim Hund für die Gesamtbildungszeit des Erythrocyten rund 3 Tage. ERSLEV (1959) konnte eine Reticulocytose beim Kaninchen nach Blutentnahme nicht vor 24—48 Stunden beobachten. Bei der perniciösen Anämie des Menschen setzt die Reticulocytose etwa am 3. Tag nach Behandlungsbeginn ein (MINOT und CASTLE 1935).

20 Tage nach der Blutung war in unseren Versuchen der Ausgangswert der Hämoglobinmenge erreicht worden. ROBSCHEIT-ROBBINS, MILLER und WHIPPLE (1945) hatten beim wesentlich stärker anämischen Hund für die Regenerationszeit 18 Tage, CARTLAND und KOCH (1928) bei der ebenfalls stärker anämischen Ratte 10—13 Tage errechnet. Ursachen für die errechnete kürzere Regenerationszeit lassen sich leicht aus den von den Autoren verwendeten Untersuchungsverfahren herleiten: Die Tiere wurden durch häufige Blutentnahme bei einer niedrigen Hämoglobinkonzentration gehalten, wobei aus der abgenommenen Hämoglobinmenge die per Zeiteinheit produzierte berechnet wurde. Die Hämoglobinproduktion nimmt jedoch mit zunehmender Gesamtmenge Hämoglobin ab, sodass Rückschlüsse von der zu Beginn maximalen Bildung auf die Gesamtbildung irreführend sind.

Die abnehmende Bildungsgeschwindigkeit bei zunehmender Gesamtmenge Hämoglobin ist auf den immer geringer werdenden erythropoietischen Reiz zurückzuführen und nicht darauf, dass sich die Vorräte der am Aufbau beteiligten Substanzen, etwa des Eisens, erschöpfen. Tiere ohne Zusatz von Eisen in der Nahrung verhalten sich unter Anämie genauso wie solche mit Eisenzusatz und sind in nachfolgender Hypoxie zu erheblich stärkerer Hämoglobinbildung als während Anämie fähig (TRIBUKAIT 1960 c).

Die Veränderungen der Hämoglobinmenge nach Blutung verdienen auch in anderen Zusammenhängen Interesse. Bei Erythrocyten-Lebenszeitbestimmungen mit C^{14}-Glycin an Ratten, die durch Blutentnahme anämisch gemacht worden waren, hatten BERLIN und LOTZ (1951) angenommen, dass »little, if any, of the decrease in the specific activity of the hemoglobin after day 10 can be attributed to dilution resulting from an increased total red cell volume«. Die von den Autoren berechnete Lebenszeit der neugebildeten Zellen von 27 Tagen dürfte deshalb zu kurz sein.

Auf die Schwierigkeiten, die der Unterschied zwischen Körperhämatocrit und Hämatocrit des Zentralblutes bei der Berechnung des Blutvolumens mit sich bringt, ist an anderer Stelle eingegangen worden (TRIBUKAIT 1960 b). Nach Untersuchungen von CHAPLIN, MOLLISON und VETTER (1953) sowie HUGGINS, SMITH und DEAVERS (1957) ist während Anämie das Verhältnis Körperhämatocrit/venöser Hämatocrit gegenüber Normalverhältnissen unverändert. In eigenen Versuchen lag bei 18 Anämietieren in Übereinstimmung mit Normaltieren die Hämoglobinkonzentration des Schwanzblutes gegenüber dem Zentralblut um 12.4 ± 1.44 % höher. Es wird deshalb angenommen, dass

ein durch die Methode bedingter systematischer Unterschied zwischen der Bestimmung des Blutvolumens vor und während Anämie nicht vorliegt.

Im Gegensatz zu den vorliegenden Resultaten war bei gleichzeitig vorgenommenen Untersuchungen mit Cr^{51}-gezeichneten Erythrocyten und CO das Blutvolumen teilweise abgesunken, was möglicherweise auf ein stärkeres Trauma oder auf die etwas grössere abgenommene Blutmenge zurückgeführt werden kann (ENGSTEDT, PERIĆ, TRIBUKAIT 1960).

Die Untersuchungen wurden aus Prof. T. SJÖSTRAND zur Verfügung gestellten Mitteln der »Stiftelsen Therese och Johan Anderssons Minne« bestritten.

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Acta physiol. scand. 1960. 49. 155—164

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Selective Adequate Activation of Large Afferents from Muscle Spindles and Golgi Tendon Organs

By

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Abstract

LUNDBERG, A. and G. WINSBURY. *Selective adequate activation of large afferents from muscle spindles and Golgi tendon organs.* Acta physiol. scand. 1960. *49.* 155—164. — An apparatus has been devised for brief stretching of muscles and a method is described for selective activation of I a and I b afferents. With muscles under light initial tension all I a afferents can be activated by a brief stretch of less than 100 μ without co-activation of any I b afferents. A brief pull applied during contraction of an initially slack muscle activates I b afferents but not I a afferents. With intracellular recording from motoneurones brief stretches activating only I a fibres were found to evoke monosynaptic excitatory postsynaptic potentials almost indistinguishable from those resulting on stimulation of the muscle nerve.

The central connections of the various systems of muscle afferents have been extensively investigated. The most detailed surveys have been made with electrical stimulation of muscle nerves. It is possible to discriminate between fibres of different receptor functions because of differences in electrical threshold and conduction velocity. Of particular importance was the finding that the group I volley from nerves to thigh muscles often has two components (BRADLEY and ECCLES, 1953). Detailed investigations have shown that the majority of I a afferents (with annulo-spiral endings on muscle spindles) are in the fast low-threshold component, denoted the I a volley, and the I b afferents (from Golgi tendon organs) with few exceptions in the slow high threshold com-

ponent of the group I volley, denoted the I b volley (LAPORTE and BESSOU, 1957; ECCLES, ECCLES and LUNDBERG, 1957 a). This separation is sometimes found in nerves from ankle muscles but more often there is no difference in conduction velocity between I a and I b afferents from these muscles. The threshold difference between the I a and I b volley has been the basis for a survey of the central actions by afferents from these two receptor systems on motoneurones (ECCLES, ECCLES and LUNDBERG 1957 b, c, ECCLES and LUNDBERG 1959 a, b), on the intermediate nucleus of Cajal (ECCLES, FATT and LANDGREN 1956), on the neurones of the dorsal and ventral spino-cerebellar tracts (LUNDBERG and OSCARSSON 1956, OSCARSSON 1956, 1957).

In most instances the usage of the I a and I b volley permits a satisfactory differentiation between I a and I b effects but there are occasions when an even more highly discriminative activation would be useful. A number of workers have attempted to use adequate activation of muscle receptors to differentiate between I a and I b actions, basing discrimination on differences in sensitivity to stretch or on the fact that the I a afferents pause, whereas the I b afferents discharge during contraction. However, the possibility that the receptor function of the small muscle afferents may overlap with those of group I a and I b cannot be excluded and, in our opinion, central actions adequately evoked from muscle can be ascribed to group I only if they occur after such a brief delay that conduction in small afferents is thereby excluded. In the present investigation a method has been worked out whereby, among group I afferents, selective activation of either I a or I b afferents can be achieved through a brief stretch of the muscle which gives a highly synchronous afferent volley permitting accurate measurements of central delays.

Methods

The experiments were made on spinal cats under light anaesthesia from pentobarbitone sodium. The ventral roots were sectioned and the activity was recorded in thin dorsal root filaments containing one or a few afferents from soleus. Gastrocnemius and plantaris were removed to expose the soleus muscle. The nerve to soleus was dissected for stimulation but left in intact connection with the muscle. Intracellular recording from motoneurones was made as described by BROCK, COOMBS and ECCLES (1952).

A diagram of the apparatus used to apply a brief stretch to the soleus tendon is shown in Fig. 1. A sliding slug A, when released by a press switch, tripped in turn a microswitch C and the lever D. C triggered the sweep and the stimulators after variable delays and the lever system D was connected to the muscle tendon by a light steel rod.

The slug A, which slid freely in a slotted tube G, was attached to the lead weight E and when released the slug was induced to move with an acceleration of approximately 25 m/sec per sec. Two winding drums and gears with a 3: 1 ratio were interposed between the slug and the lead weight in order to increase acceleration.

After operation the weight E was lifted into position by means of a DC motor driving a winding drum K via an electromagnetic clutch L. When the weight E again was in

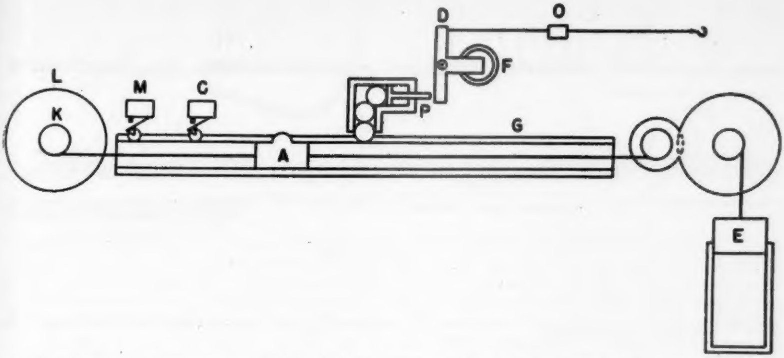

Fig. 1. Diagram of apparatus designed for brief stretching of the muscle. See text.

position the slug A actuated a microswitch M, which reduced the current through the motor armature and the electromagnetic clutch to such a value that the weight E was just held in position.

The lever system D consisted of two levers mounted on a common shaft. Attached to one lever was a rod leading to a strain gauge O and the muscle tendon. A metal shutter was attached to the same lever and served to control the output from a photocell F whereby the movement of the lever was recorded. The other lever was struck by a plunger P which could be moved nearer to or away from the lever by means of a micrometer; so altering the distance through which the lever moved. The plunger was actuated by the impact of three 6 mm steel balls, the first of which was struck by the slug A as it slid down the slotted tube G. Movement of the first ball caused a second ball to move in a horizontal direction causing the plunger to strike the lever system D. The change of direction of the steel ball gives an increased initial displacement of the plunger.

The strain gauge O was connected between the muscle tendon and the steel rod leading to the lever D. The gauge consisted of a zig-zag of insulated 50 s. w. g. nichrome wire woven in one plane into rayon thread and rolled up. It formed part of a double Kelvin bridge.

The assembly in Fig. 1 was built up on a heavy welded steel base with adjustable legs.

Results

Experiments with activation of muscle afferents by maintained stretch have demonstrated that the threshold for I a fibres is considerably lower than for I b fibres (MATTHEWS 1933, HUNT and KUFFLER 1951). In addition spindle afferents respond more effectively to phasic stretch than tendon organ afferents (MATTHEWS 1933). With these two facts in mind we have tried if it is possible by a brief slight stretch of muscle to activate the I a afferents synchronously without stimulating the I b afferents. The records in Fig. 2 were obtained from a I a fibre from soleus. A shows the action potential caused by stimulation of

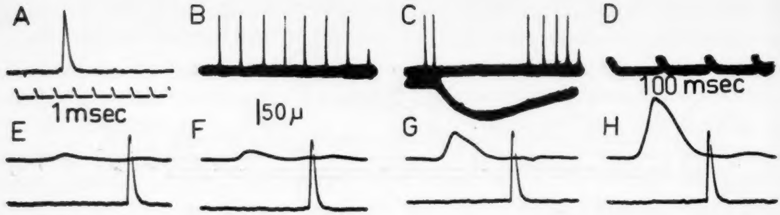

Fig. 2. Activation of Ia afferents from soleus. Recording was made from a dorsal root filament. A shows the effect of electrical stimulation of the soleus nerve and E—H, obtained with the same sweep speed, of brief stretches applied to the soleus tendon. The upper traces in E—H are the photoelectrically recorded time course of the stretches delivered by the apparatus. In B and C at slower sweep speed the muscle was stretched to evoke a background discharge with C showing the pause during contraction.

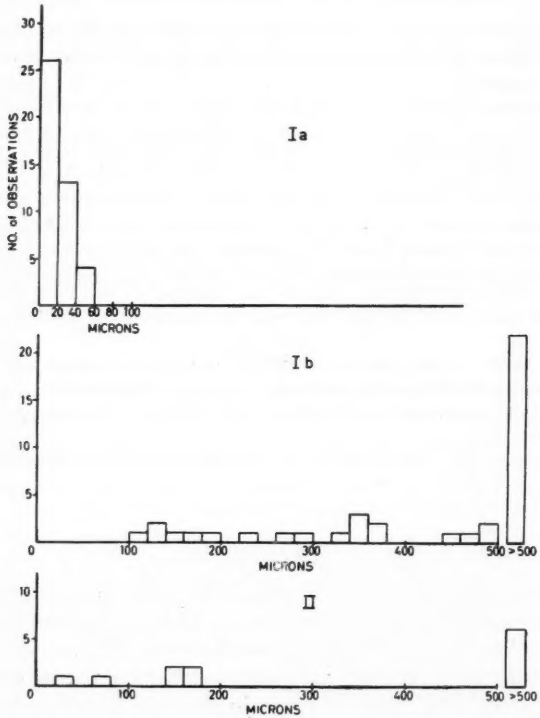

Fig. 3. Distributions of thresholds for brief-stretch activation of I a, I b and group II afferents from soleus. Recording was made from thin dorsal root filaments and the afferents classified according to conduction velocity and behaviour during contraction. The soleus muscle was kept under a tension of a few grams.

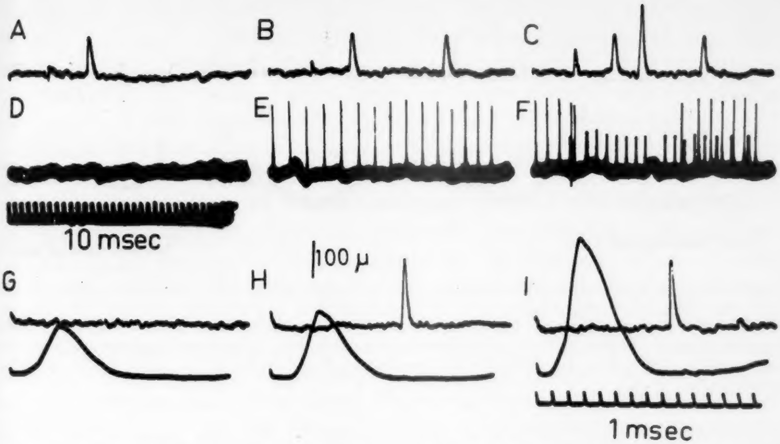

Fig. 4. Recording from dorsal root filament containing a I b and a group II fibre from soleus. A—C show the effect of electrical stimulation at increasing strength of the soleus nerve. In D at zero tension there was no resting discharge. In E the soleus tendon was pulled to evoke a discharge in the group II but not in the group I b fibre. During contraction in F a discharge appears in the I b fibre, whereas the discharge in the group II fibre pauses. In G—I brief stretches were applied, resulting in excitation (H and I) in the group II but not in the I b fibre.

the nerve to soleus, B the steady discharge caused by a slight stretch and C the pause during contraction. Brief pulls were applied to the tendon of soleus in E—H. In E the pull was smaller than 10 μ but nevertheless gave rise to a spike. With a stretch of 20 μ (F) the latency shortened by about 1 msec, but with further increase of the stretch the latency did not shorten more than a 0.1—0.2 msec. Altogether 43 I a afferents were investigated and the results are summarized in Fig. 3 (upper graph). The largest stretch ever needed for activation was 60 μ and the large majority of the fibres could be activated by a pull of less than 25 μ. A moderate pull never evoked more than a single spike, a double discharge was found only when the muscle was stretched more than 1 mm and sometimes not even then.

The middle graph in Fig. 3 summarizes the corresponding results on I b afferents. None of the I b fibres tested was activated by a stretch of less than 100 μ and only 6 out of 41 by a stretch of less than 200 μ (cf. Fig. 4). Some could not be activated even with a stretch of 1.5 mm which was the maximum delivered by our apparatus. In summary it can be concluded that at low initial tension stretch of the tendon not exceeding 100 μ evokes a maximal I a volley without activation of any I b afferents.

Stretch activation was also investigated with some group II afferents. The records in Fig. 4 were obtained from a filament containing one I b fibre (conducting at 83 msec) and one group II fibre (conducting at 50 msec). At

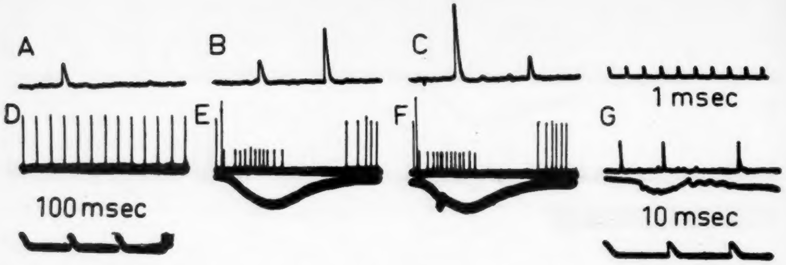

Fig. 5. Recording from dorsal root filament containing a I a and I b fibre from soleus. A—C show the effect of electrical stimulation of the soleus nerve at increasing strength. The I a discharge in C was evoked by a slight stretch of the tendon and record E shows that the small spike is from a I b fibre, the large one from a I a fibre. In F a pull was superimposed on the contraction; there is no I a discharge but an extra I b spike elicited as shown better at faster sweep speed in G.

weak stimulation the I b spike appears alone in A. In B at increased strength the alpha efferents were stimulated and an early discharge appears in the I b fibre (Hunt and Kuffler, 1951) and with further increase of stimulus strength in C the group II fibre was activated. Brief stretches were applied to the soleus tendon in G—I. A stretch of 1 mm was needed for activation of the I b fibre but the group II fibre was stimulated by stretches exceeding 160 μ (H). The latency of the stretch-evoked spike in I is 3.75 msec longer than in C with electrical stimulation of the soleus nerve and similar differences were found with the other group II fibres investigated. The corresponding difference in Fig. 2 for a I a fibre was 1.6 msec. This difference of more than 2 msec is probably only partly due to the differences in conduction velocity, presumably initiation of the spike is somewhat slower in group II than in I a fibres. All factors considered the stretch evoked group II volley will reach the spinal cord 3—4 msec later than the group I a spike, as compared with 1—2 msec on electrical stimulation of the nerve.

In Fig. 3 (lower graph) the threshold for activation by brief stretches are shown for 12 group II fibres; 2 of them were activated by a stretch of less than 100 and 6 needed a stretch of more than 500 μ. Hence it is clear that even if group II fibres have some overlap with I a, the average threshold for activation by brief stretch is much higher. In this connection it should be remembered that Hunt (1954) found that group II have a higher average threshold for maintained stretch than the fibres of group I a (mean values 19 and 3 g respectively). Also in our experiments the average threshold for maintained stretch was markedly higher for group II. Hunt states in his paper that I a and II display qualitatively similar responses to phasic stretch. On the other hand, Cooper (1959) found that in group II fibres the rate of discharge during the application of stretch hardly exceeds that reached during a maintained stretch,

whereas the I a fibres respond with very rapid rates during the dynamic phase of stretch. The results of our experiments with manual stretching of the soleus tendon agree entirely with those of Cooper; at the onset of stretch group I a but not group II respond with a burst of impulses. Hence it is not surprising that group II fibres have a higher threshold for brief stretches than I a.

It is also possible to apply a pull activating tendon organ afferents but not spindle afferents. This is achieved if the pull is applied when the muscle contracts. Fig. 5 shows records from a dorsal root filament with one I a and one I b fibre from soleus. The conduction velocities were identical for both fibres (record C), the electrical thresholds were in the upper range overlapping with the alpha efferents. The Golgi afferent was electrically stimulated in record A and an early discharge evoked in the spindle afferent (B). In record C the stimulus strength had been raised to excite also the spindle afferent; now there is an early discharge in Golgi but not in the spindle afferent. In records D—F a steady very slight stretch was applied to the tendon in order to make the I a afferent discharge with the frequency seen in D. With contraction of the muscle this discharge pauses in E and the discharge in the I b afferent appears. In F a brief pull is applied to the tendon during the rising phase of the contraction as can be seen in the myogram. It causes an extra I b spike but no I a discharge. The stretch-initiated spike is seen at higher sweep speed in G to occur after a latency of 3.7 msec, compared with 1.8 msec for the spike in C. In order to ensure selective activation of I b afferents by a rapid stretch it is necessary to apply the stretch in a relatively early phase of contraction. If the stretch is applied later than in F, Fig. 5 I a afferents may be activated as well. The reason for this is unknown; to evoke contraction the soleus nerve was stimulated below γ-strength. However, one possibility to consider is activation of larger spindle efferents (cf. GRANIT, POMPEIANO and WALTMAN 1959 a, b, BOYD and DAVEY 1959).

In Fig. 5 the stretch-initiated I b volley is evoked during a background discharge of 100/sec, caused by the contraction itself. It is possible to decrease or abolish this background discharge by using an initially slack muscle whereby less tension is developed during contraction. Nevertheless a superimposed rapid stretch evokes a I b volley (cf. LUNDBERG and WINSBURY 1960 Fig. 1) but in this case not in all I b fibres. Some fibres which did not respond to a steady pull of more than 1 kg were not activated by a pull applied during contraction of an initially slack muscle. It is by this method possible to obtain a synchronous volley in about 75 % of the I b afferents and with relatively little background discharge. On the other hand by applying a rapid pull to a muscle contracting against a slight stretch it is possible to evoke a fairly synchronous volley in all the I b afferents, but in this case contraction by itself evokes a relatively heavy background discharge.

Intracellular recording from motoneurones was used to study the synaptic actions evoked by brief stretches. The records in Fig. 6 were obtained from a

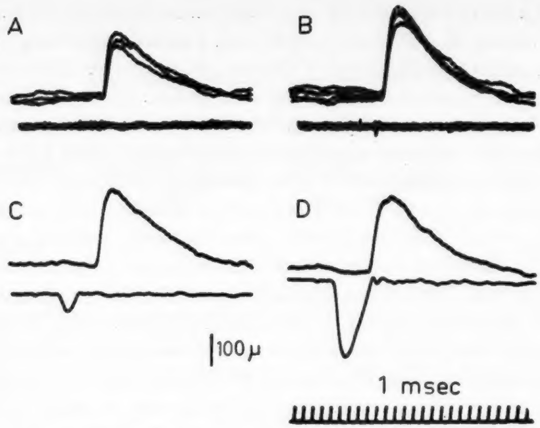

Fig. 6. Intracellular records from a motoneurone identified as belonging to soleus because of its pattern of I a convergence (from gastrocnemius and plantaris) and its longlasting after-hyperpolarization. The excitatory post-synpatic potentials in A and B were evoked on stimulation of the soleus nerve; those in C and D by brief stretches applied to the soleus tendon. About 5 traces were superimposed in A and B, single traces were photographed in C and D.

soleus motoneurone on stimulation of the nerve to soleus (A and B) and by applying a brief stretch to the soleus tendon (C and D). The monosynaptic EPSP in B was maximal but stimulation was submaximal to the alpha efferents so there was no synaptic action due to the early discharge. In C a pull of about 50 μ gives an EPSP of almost the same time course and size as that evoked on electrical stimulation of the nerve. It is not significantly changed in D when a stretch of 250 μ is applied to the tendon. The latency difference between the stretch evoked EPSP and the EPSP evoked on stimulation of the soleus nerve is 1.5 msec which corresponds very well to the difference of 1.6 msec in Fig. 2, measured on primary afferents; in both cases the distance from the stimulating cathode to the middle of the muscle was 70 mm. The similarity in time course of the EPSP's in A—B and C—D illustrates well how synchronous the stretch evoked volley in the primary afferent may be. The records in Fig. 6 are the intracellular correlates of the monosynaptic reflexes recorded by LLOYD (1943) in response to brief stretch of muscle, except that in his experiments, in which the stretch was not quite as brief as in ours, there was evidence of multiple discharge.

It was of particular interest to investigate the stretch-evoked synaptic actions in soleus motoneurones since the method of selective stretch-activation was worked out with afferents from this muscle. These experiments therefore provide a conclusive proof that the monosynaptic EPSP in motoneurones is caused by afferents with annulo-spiral endings on muscle spindles. Similar

records as those in Fig. 6 were obtained from motoneurones of gastrocnemius and flexor digitorum longus on stretching these muscles. An attempt was also made with intracellular recording from motoneurones to study I b actions resulting from pulls applied during contraction, but fixation of the animal was not sufficiently rigid to allow reliable measurements of the membrane potential during contraction of the muscle.

Discussion

It was known that I a fibres with annulo-spiral endings on muscle spindle have a lower threshold for stretch than I b fibres, from Golgi tendon organ, and furthermore that they respond more easily to phasic stretch than I b afferent (MATTHEWS 1933, HUNT and KUFFLER 1951). It has now been shown that if a brief pull lasting for only a few milliseconds is applied to the tendon of a muscle kept at low initial tension one spike can be evoked in all I a afferents without concomitant activation of any I b fibres. By this method some differentiation can be achieved also between I a and group II afferents. At a pull of 100 μ which is well above what is needed for activation of all I a afferents less than 20 % of the group II afferents responded. On the other hand a brief pull during contraction of an initially slack muscle gives a spike in the majority of the I b fibres but not in the muscle spindle afferents. Activation of all I b afferents by a brief pull is achieved if the muscle is under some initial tension, but in this case there is a rather heavy background discharge evoked by the contraction itself.

As with other methods for adequate stimulation of muscle receptors coactivation of small afferents cannot be excluded. However, it should be noted that synchronous volleys are evoked by these brief pulls; hence it should in most cases be possible to decide from latency measurements if a central action evoked by such a brief pull is caused by impulses in group I afferents.

The present experiments have therefore provided a new method to differentiate between I a and I b actions. This method is undoubtedly more cumbersome than the usage of the I a and I b volley evoked by electrical stimulation of muscle nerves, but it is more selective and may be used supplementarily to this method in crucial instances. The employment of the method in the study of central actions may be examplified by the experiments with intracellular recording from motoneurones. The monosynaptic EPSP evoked by a pull of less than 100 μ is almost indistinguishable from the one resulting on electrical stimulation of the nerve. It has already been proved that impulses in I a afferents are responsible for the monosynaptic EPSP (ECCLES *et al.* 1957 a, LAPORTE and BESSOU 1957); the present results provide confirmation by an independent method.

Technical assistance was given by Miss RUTH ARALDSSON.

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Functional Organization of the Dorsal Spino-Cerebellar Tract in the Cat.

VI. Further Experiments on Excitation from Tendon Organ and Muscle Spindle Afferents

By

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Abstract

Lundberg, A. and G. Winsbury. *Functional organization of the dorsal spino-cerebellar tract. VI. Further experiments on excitation from tendon organ and muscle spindle afferents*. Acta physiol. scand. 1960. *49*. 165—170. — Recording was made from fibres in the dorsal spino-cerebellar tract activated by group I afferents from soleus. Adequate activation through brief stretches giving selective activation of I a or I b afferents was used to differentiate between units activated by I a and I b afferents. There was no evidence of convergence of excitatory action by I a and I b afferents to the same neurones. The receptive field is often larger for neurones activated by spindle than for neurones activated by tendon organ afferents.

In previous papers of this series two functional subgroups of the dorsal spino-cerebellar tract (DSCT) were identified. It was concluded that some DSCT neurones subserve transmission from muscle spindles (I a and II) and others from Golgi tendon organs (I b) (Laporte, Lundberg and Oscarsson 1956, Laporte and Lundberg 1956, Lundberg and Oscarsson 1956). However, some findings were made which could suggest convergence of excitatory action from muscle spindles and Golgi tendon afferent to the same DSCT neurones. It was observed that in DSCT neurones excited by a I a volley a second post-synaptic spike appeared with a I b volley generated in the refrac-

tory period of the preceding I a volley. However, this action by the I b volley may not necessarily be a genuine action by I b fibres. It has been shown that there may be some intermixture between the I a and the I b volleys; the slow high threshold component of the group I volley may contain a few I a afferents and vice versa there may be some I b fibres in the fast high threshold component (Laporte and Bessou 1957, Eccles, Eccles and Lundberg 1957). It is also known that on synaptic activation of Clarke's column cell some monosynaptic excitatory action survives the excitation (Curtis, Eccles and Lundberg 1958) and actually may reduce the membrane potential enough to generate a second action potential (cf. Laporte *et al.* 1956). In the situation previously described, when a second impulse was evoked by a I b volley generated in the refractory period of a preceding I a volley, it should be considered that this occurred when the excitability of the post-synaptic membrane was high; a few intermixed I a fibres in the I b volley may have sufficed to generate an impulse (cf. Eccles *et al.* 1957).

In the present investigation the new method for selective adequate activation of I a and I b afferents (Lundberg and Winsbury 1960) has been applied and no evidence has been found indicating convergence of excitatory action form I a and I b afferents to the same DSCT neurone.

Methods

The experiments were made on spinal cats under light anaesthesia from pentobarbitone sodium. Laminectomy was made in the lower lumbar region for recording of the incoming volley and in the lower thoracic region for intra-axonal recording from DSCT fibres as described by Laporte *et al.* (1956). Adequate activation of receptors in soleus through brief stretches was made as described by Lundberg and Winsbury (1960). The nerves to plantaris, flexor digitorum longus and medial gastrocnemius were also dissected, but were severed from the muscle.

Results

The procedure was to identify axons in the dorsal part of the lateral funicle which could be activated by a group I volley evoked by electrical stimulation of the soleus nerve. These units were tested with respect to adequate activation from soleus. A discharge was evoked by pulling the soleus tendon and the units could be classified as forwarding spindle or tendon organ information depending on whether they paused or accelerated during contraction of the muscle. The records in Fig. 1 are from a tendon organ unit. The soleus nerve was stimulated at threshold for activation of the DSCT unit in A and at higher strength, including also the alpha efferents, in B leading to a second spike (the early discharge). This unit had a resting discharge of a few per sec., it responded to a maintained stretch in D and accelerated in E when made to contract against this maintained stretch. On the other hand, when the muscle

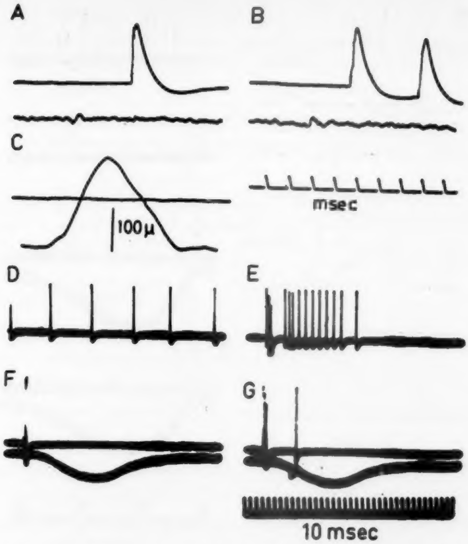

Fig. 1. Absence of I a excitation in neurone excited by I b afferents. Intra-axonal recording in LI from DSCT axon. Record A and B show the effect of electrical stimulation of the soleus nerve at increasing strength. The lower traces show the incoming volley recorded at the dorsal root entry zone. In C a brief stretch was applied to the soleus tendon (initial tension about 3 g), with the lower trace showing the photo-electrically recorded time-course of the stretch delivered by the apparatus. The discharge in D was obtained on manual stretching of the tendon and in E the muscle contracted against this maintained stretch. The contraction in F took place in a muscle initially about 15 mm shorter than zero length (the length to which a muscle can be stretched without development of tension). In G a pull was superimposed on the contraction. The contractions were evoked by stimulation of the soleus nerve at a strength just maximal for the alpha efferents.

was slack initially (about 150 mm short of resting length) contraction by itself did not evoke a discharge in F, but a superimposed brief stretch during contraction did elicit a spike (G) as would be expected in a neurone activated by Golgi tendon organ afferents.

It was now possible to test if this neurone received subsidiary excitation from I a fibres by applying a brief stretch to the resting muscle kept under light initial tension. A brief stretch of 200 μ did not elicit a spike in C. This stretch is more than sufficient to excite synchronously all I a fibres (Lundberg and Winsbury 1960), hence there is no indication of a subsidiary I a excitation to this neurone. 14 neurones activated by Golgi tendon organ afferents from soleus were tested with the same result.

Conversely it was also tested if DSCT neurones activated from spindle afferents received subsidiary excitation from tendon organ afferents. The records in Fig. 2 were obtained from a spindle-unit; the discharge from group I

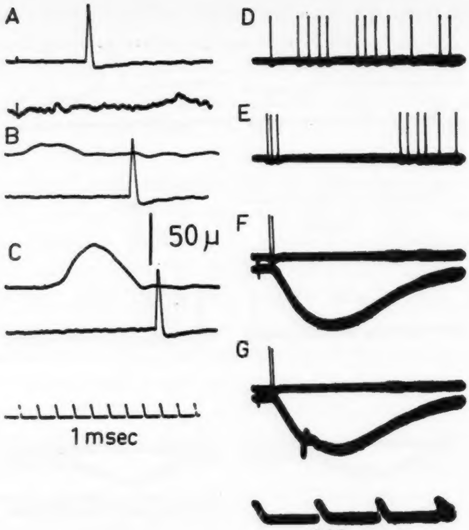

Fig. 2. Absence of I b excitation in neurone excited by I a afferents. Intra-axonal recording in LI from DSCT axon. Record A shows the effect of electrical stimulation of the soleus nerve, B and C the effect of brief stretching of the soleus muscle. The discharge in D was evoked by a slight maintained stretch of the muscle and with contraction there is a pause in E. In F and G the muscle contracted from zero initial length. The superimposed brief pull in G does not evoke a spike. The contractions were evoked by stimulation of the soleus nerve at a strength just maximal for the alpha efferents.

stimulation of the soleus nerve is shown in A; a pause during contraction of a stretched muscle in E. The brief stretches in B and C, 10 and 40 μ respectively, excited the neurone as would be expected in a unit excited by spindle afferents. On the other hand in F when a brief pull was superimposed on the contraction there was no excitation, so there is no indication of any subsidiary I b excitation to this spindle unit. 15 DSCT neurones activated by I a afferents from soleus were investigated with the same result.

Holmqvist, Lundberg and Oscarsson (1956) and Curtis *et al.* (1958) demonstrated that the receptive field for group I activated DSCT neurones is small. Many neurones could only be excited from one muscle, others from two or three. In the present experiments we have taken the opportunity to investigate if there is any difference in this respect between neurones excited by tendon organ and spindle afferents. Effects from the nerves to plantaris, flexor digitorum longus (FDL) and medial gastrocnemius were investigated in 13 neurones excited by I b afferents from soleus. None of these neurones could be excited from any of these other nerves, but two of them received subliminal

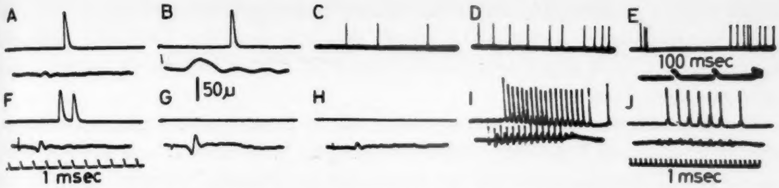

Fig. 3. Convergence on to DSCT neurone activated by I a afferents from soleus. Stimulation of the nerves to soleus A, plantaris F, flexor digitorum longus G and medial gastrocnemius H. Record B shows the effect of brief stretching of the soleus muscle. The resting discharge with the muscle slack is shown in C, in D is shown the effect of a maintained stretch of the soleus tendon and in E the pause during contraction of soleus. Transmission of a train of volleys from the plantaris nerve is shown in I and from the soleus nerve in J.

excitation from plantaris, as evidenced by a facilitatory action from a group I plantaris volley on a soleus volley just subliminal for excitation of the DSCT neurone.

With the units activated from spindles on the other hand there was frequently evidence of convergence. Out of 16 units activated by I a afferents from soleus 9 could be excited from at least one of the other three nerves and with some of the remaining 7 neurones subliminal excitatory action gave evidence of convergence. The records in Fig. 3 are from a DSCT unit excited by spindle afferents from soleus. The spike in A was evoked on electrical stimulation of the soleus nerve and B shows the response to a brief stretch of the soleus tendon. This neurone had a resting discharge when the soleus muscle was slack (C), on moderate stretch of soleus the frequency increased (D) and during contraction there was a pause (E). A volley from the plantaris nerve evoked a double spike response (F), but there was no excitation on stimulation of the nerve to flexor digitorum longus (G) or medial gastrocnemius (H). On repetitive stimulation the neurone followed high frequencies (800/sec) from the plantaris (I) and initially it followed 500/sec from the soleus nerve (J). Apparently plantaris was in the centre of the receptive field for this neurone, but the linkage from soleus was strong enough to permit transmission of significant effects during stretch (D—E).

Discussion

By employment of a new method (Lundberg and Winsbury, 1960) it has been confirmed that one DSCT subgroup is activated by I a afferents and another by I b afferents (Lundberg and Oscarsson 1956). As mentioned in the introduction some previous findings raised the question if excitatory action from I a and I b afferents did converge to the same DSCT neurones. With our new method, providing for a completely selective activation of I a or I b afferents, it was not possible, in any of the 29 tested DSCT neurones, to demon-

strate such a convergence. This test is more stringent than those previously applied and it may therefore be stated that the available evidence suggests independence on the excitatory side of the DSCT lines conveying spindle and tendon organ information.

Some new information has also been gained regarding the pattern of convergence to group I activated DSCT neurones. Previously it was known that many DSCT neurones are excited only from one muscle, whereas others have a limited convergence from a few muscles (Holmqvist *et al.* 1956, Curtis *et al.*, 1956). It has now been found that a wider receptive field is common with the spindle but not with the Golgi-units. It seems reasonable that during physiological activation of stretch receptors in muscle a higher degree of spatial discrimination would be more useful with respect to tendon organ than with spindle information.

Technical assistance was given by Miss Ruth Araldsson.

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Functional Organization of the Ventral Spino-Cerebellar Tract in the Cat

III. Supraspinal Control of VSCT Units of I-Type

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Abstract

Oscarsson, O. *Functional organization of the ventral spinocerebellar tract in the cat. III. Supraspinal control of VSCT units of I-type.* Acta physiol. scand. 1960. *49.* 171—183. — Ventral spino-cerebellar tract (VSCT) neurones monosynaptically activated by Golgi tendon organ afferents and with inhibitory action from group II and III muscle afferents, skin and high threshold joint afferents, have been investigated in decerebrate cats. In the decerebrate preparation the inhibitory actions are depressed due to a tonic inhibitory control of the interneurones mediating these actions. It is shown that this control changes the message forwarded by the VSCT neurones from one which has been interpreted as exteroceptive or informative of flexion reflex patterns to one which is proprioceptive.

In addition a control system not tonically active in the decerebrate preparation is described. It can be activated by stimulation of the brain stem and the responsible fibres are located in the dorsal part of the lateral funicle. It inhibits the ipsilateral VSCT neurones.

A majority of ventral spino-cerebellar tract (VSCT) neurones monosynaptically excited by Golgi tendon organ (Ib) afferents in addition receive inhibitory action from group II and III muscle afferents and from skin afferents. In the spinal state adequate stimulation of muscle receptors regularly causes inhibition of these VSCT neurones even when the stimulation is such as to evoke strong and, as far as possible, selective activation of Golgi tendon organs. The mono-

synaptic excitation from Ib afferents is concealed by inhibition from high threshold muscle afferents activated concomitantly with the tendon organ afferents (OSCARSSON 1957 a). In the previous investigation no clue was obtained as to the functional significance of the monosynaptic connection with the Ib afferents. Strong effects from cutaneous receptors made it likely that the VSCT forwarded exteroceptive information.

It has recently been shown that the inhibition of VSCT neurones is less pronounced in the decerebrate than in the spinal animal (HOLMQVIST, LUNDBERG and OSCARSSON 1960). This is due to a tonic control of the interneurones mediating the inhibition. The centres responsible for the control are located in the brain stem and the fibres descend in the dorsal part of the lateral funicle.

This finding made it of interest to investigate the effects of adequate stimulation of muscle and skin receptors in the decerebrate preparation. It will be shown that in the decerebrate animal the monosynaptic excitatory action from Golgi tendon organ afferents dominates over the inhibitory effects from group II and III afferents and that the effects from skin receptors are strongly depressed.

In addition a new control system has been discovered. Its fibres are located in the dorsal part of the lateral funicle and it is not tonically active in the decerebrate preparation. It has an inhibitory action which is exerted on the ipsilateral VSCT neurones.

Methods

The experiments were performed on unanaesthetized cats. The animals were operated on under ether anaesthesia and then decerebrated. Flaxedil was given in experiments with electrical stimulation of descending tracts.

As a routine the femoral and obturator nerves and all branches (except that to the quadratus femoris) of the sciatic nerve were severed bilaterally. In experiments with adequate stimulation of muscle receptors the right triceps nerve, and in experiments with adequate stimulation of cutaneous receptors the right and left saphenous, sural, lateral skin (traversing the biceps muscle), and superficial peroneal nerves were left intact. In some experiments the right L7 + S1 ventral roots were dissected and the distal ends stimulated for contraction of the triceps muscle. The gluteal and quadratus femoris muscles were not denervated and co-contracted.

All records were obtained with 3—5 superposed sweeps.

For further details concerning experimental technique see LAPORTE, LUNDBERG and OSCARSSON (1956 a, b) and OSCARSSON (1957 a).

Type of VSCT units investigated

VSCT units have been shown to be monosynaptically activated by Golgi tendon organ afferents (OSCARSSON 1956). The great majority of these units, the VSCT units of I-type, in addition receive inhibitory action from group II and III muscle afferents and from skin afferents. The remainder, the VSCT units of E-type, are excited by these afferents (OSCARSSON 1957 a). In a recent investigation VSCT units were identified by antidromic stimulation of their terminals in the cerebellar cortex (LUNDBERG and OSCARSSON, unpublished). By this method of identification it was found that a considerable

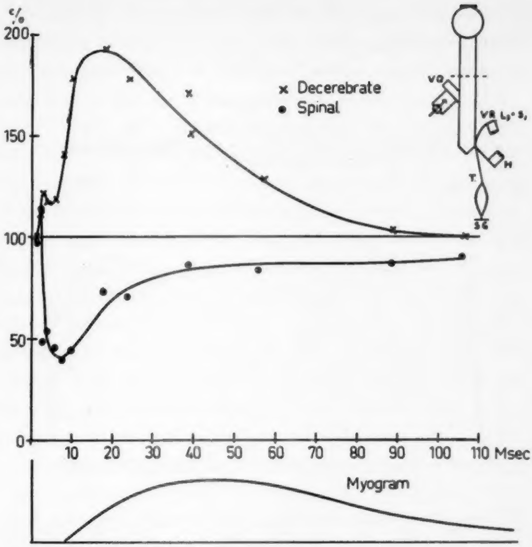

Fig. 1. Effect on VSCT neurones of isometric contraction in decerebrate and spinal preparation. The diagram shows stimulating and recording arrangements: VQ, dissected ventral quadrant at L1. VR L7 + S1, distal ends of severed L7 and S1 ventral roots. H, hamstring nerve. T, triceps nerve with muscle. SG, strain gauge connected to muscle at a tension of 500 g. Upper curves show effect of ventral root stimulation on monosynaptic VSCT response evoked by a submaximal group I stimulus to the hamstring nerve. Ordinate: Conditioned test response in per cent of unconditioned one. Abscissa: interval between conditioning and testing stimulus. The curves were obtained before and after transection of the cord at Th 13 (broken line in diagram). Curve marked 'myogram' shows time course of contraction on same abscissa as the two main curves.

number (approximately 50 per cent) of the VSCT units could not be monosynaptically activated from any of the dissected hindlimb nerves (the quadriceps nerve and all branches of the sciatic nerve). These neurones may have been connected with Ib afferents in other nerves but another possibility is that these neurones constitute a subdivision of the VSCT characterized by the lack of Ib excitation. The present investigation has been limited to VSCT neurones of I-type.

Results

1. Suprasegmental control of interneurones

a. Adequate stimulation of muscle receptors

In the spinal preparation strong isometric contraction effectively activating Golgi tendon organs causes inhibition of the VSCT neurones which receive Ib excitation from the contracting muscle. Inhibition from high threshold muscle afferents activated concomitantly with the Ib afferents conceals the monosynaptic excitation (Oscarsson 1957 a). Similar experiments have now been performed in the decerebrate animal and it will be shown that in this preparation the VSCT neurones may be excited during contraction.

The effect of contraction on VSCT neurones was tested by a submaximal Ib volley. In most experiments a volley from the hamstring nerve was used for testing the effects following contraction of the triceps muscle. This was possible because of a marked convergence of Ib excitation from the hamstring and

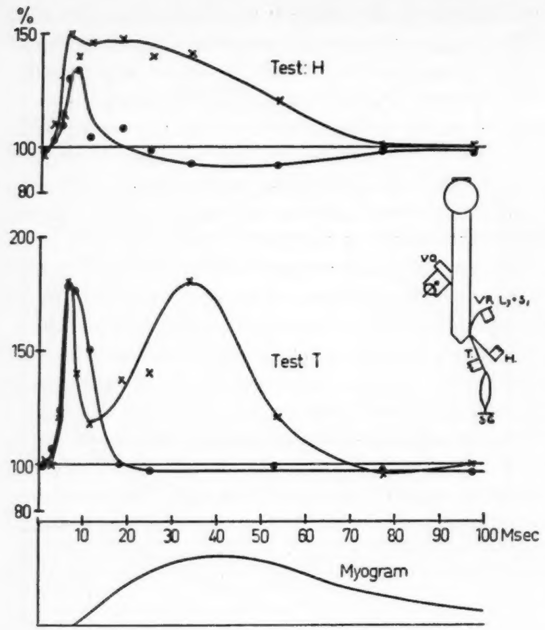

Fig. 2. Comparison of effect on isometric contraction and contraction without resistance. The diagram shows stimulating and recording arrangements. Abbreviations and lowermost curve as in Fig. 1. The two pairs of curves show effects of ventral root stimulation on submaximal monosynaptic response evoked from hamstring nerve (upper curves) and from triceps nerve. Ordinate: conditioned test response in per cent of unconditioned one. Abscissa: interval between conditioning and testing stimulus. Crosses indicate values obtained on isometric contraction and dots values obtained on contraction without resistance.

triceps nerves on to the same VSCT neurones. In this way artefacts due to movements of the triceps nerve could be avoided. Fig. 1 illustrates a typical experiment. Contraction of the triceps muscle was obtained by stimulation of the L7 + S1 ventral roots. This resulted in facilitation of the VSCT discharge elicited from the hamstring nerve. The first phase of facilitation was presumably due to the early discharge (HUNT and KUFFLER 1951) in tendon organ afferents from some undenervated muscles of the pelvic girdle (see Methods). The first part of the second phase is attributable to the early discharge from the triceps muscle. This part was often separated from the later facilitation by a dip as shown in Fig. 2. The facilitation reached a maximum before the contraction became maximal as is also the case with the discharge in Golgi tendon organ afferents. After spinalization the effect of ventral root stimulation changed into inhibition. This inhibition was caused by impulses in group II and III afferents from the triceps muscle but also from the undenervated proximal muscles. A slight initial facilitation was probably due to the early discharge in tendon organ afferents of the latter muscles.

As the facilitation is due to a discharge in tendon organ afferents there will be a marked difference between the effects evoked when the muscle contracts isometrically and when it contracts without resistance. The upper curves in

Fig. 2 were obtained with the testing stimulus applied to the hamstring nerve. Isometric contraction of the triceps muscle caused a facilitation similar to that shown in Fig. 1. In this case the main part of the early phase appeared later than in Fig. 1 and was mainly due to the early discharge in Golgi tendon organ afferents of the triceps muscle. When the muscle contracted without resistance the facilitation was converted into a slight inhibition except for the early phase which remained mainly unchanged. Recording from dissected dorsal root filaments confirmed that the early discharge in tendon organ afferents remained under these conditions. The second phase of facilitation disappeared as the tendon organs were not activated by any increased tension. The slight inhibition was probably due to effects from group II and III afferents. The lower curves were obtained from the same experiment but in this case the testing stimulus was applied to the triceps nerve. The marked dip in the curve obtained on isometric contraction is presumably due to refractoriness of the primary afferents and the VSCT neurones following the early discharge.

Experiments with unit recording were performed on decerebrate cats. VSCT units identified as monosynaptically activated from Ib afferents in the triceps nerve were regularly facilitated during isometric contraction of the muscle. In some units the excitation resulted in a discharge of impulses during the rising phase of the contraction. VSCT units identified as monosynaptically activated by Ib afferents of the hamstring nerve behaved differently according to whether they received Ib excitation from the triceps nerve or not. Those which had a fairly strong connection with Ib afferents in the triceps nerve were facilitated during isometric contraction, those which had no connection, or a weak one, were not influenced or slightly inhibited.

In most units one or two spikes appeared with a short latency on stimulation of the ventral roots L7 + S1. The first spike had a latency of 3.9 to 5 msec and corresponds to the early phase of facilitation interpreted as due to the early discharge in tendon organ afferents of certain undenervated proximal muscles. The second spike appeared in those units which had Ib connection with the triceps nerve. It had a latency of 5.5 to 7.5 msec and was due to the early discharge in Ib afferents of the triceps muscle. These early spikes in the VSCT units were usually evident as a mass discharge on recording from the ventral quadrant. In Fig. 3 stimulation of the ventral roots characteristically evoked a discharge with two peaks. For comparison the discharge evoked by direct stimulation of Ib afferents in the triceps nerve is shown. As in most experiments the contraction did not evoke any marked mass discharge. This is consistent with the finding that most of the VSCT neurones did not discharge during contraction.

Two factors are relevant when considering the often fairly weak excitatory effect in the VSCT neurones during isometric contraction. 1.) In the decerebrate preparation the Ib excitation is partly concealed by inhibitory action from group II and III muscle afferents. It is not unlikely that the control of the interneurones

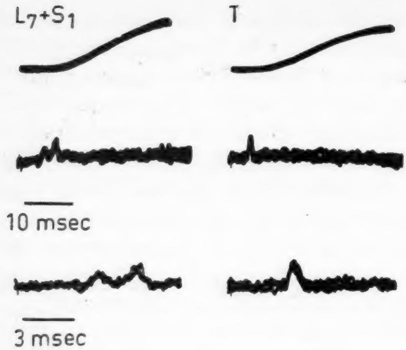

Fig. 3. Transmitted early discharge in VSCT neurones. Stimulating and recording arrangements as in Fig. 2. Left row of records obtained on stimulation of the ventral roots and right one on stimulation of the triceps nerve. The contraction was isometric. Upper records show beginning of myogram. Middle and lower records show the discharges evoked in the ventral quadrant on slow and fast time scale. *Note*: In contrast with the case in some other experiments there was no second discharge signalling the early discharge in tendon organ afferents when the triceps nerve was stimulated. Probably the tract neurones were refractory after the discharge caused by the Ib volley.

mediating the inhibition may, under appropriate conditions, be more effective than it is in the decerebrate preparation. 2.) There is usually convergence of Ib excitation from several muscle nerves on to the same VSCT neurone (Oscarsson 1957 a). Probably some of the investigated units had a stronger coupling with Ib afferents in other nerves than the triceps nerve. Furthermore, it is possible that the VSCT units normally are activated by the simultaneous discharge of Golgi tendon organ afferents from several muscles (see Discussion).

b. Adequate stimulation of skin receptors

In the spinal animal stimulation of cutaneous receptors strongly affects the VSCT neurones (Oscarsson 1957 a). In the decerebrate preparation only weak effects from the skin were obtained. In one experiment 11 VSCT units activated monosynaptically from Ib afferents in either the hamstring or quadriceps nerve were investigated. Inhibitory effects could be evoked from skin areas of the contralateral hindlimb as has been described for units in the spinal preparation. However, these effects were slight and could only be evoked by strong pinching. Excitatory effects could be evoked in only two units. These excitatory effects were weak and obtained on pinching from a small skin area. The animal was then spinalized and 12 further units identified. The behaviour of these units was similar to that described previously for VSCT units in the spinal animal. Strong inhibitory effects were evoked on slight touch from areas of the contralateral hindlimb. In 9 of the units the inhibitory area was surrounded by a large area from which excitatory effects could be evoked on very slight touch.

Before spinalization only 1 of the 11 units had a resting activity. After spinalization 7 of the 12 units were spontaneously active. It has been suggested (Oscarsson 1957 a) that the resting activity is partly dependent on excitatory effects from the periphery. Thus units with large excitatory skin areas have

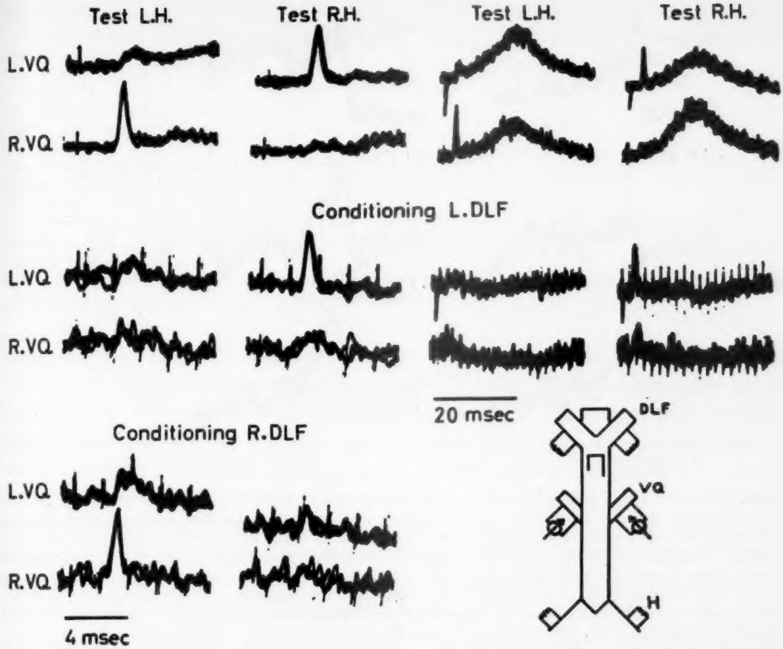

Fig. 4. Stimulation of fibres in dorsal part of lateral funicle with inhibitory action to ipsilateral VSCT neurones. The diagram shows stimulating and recording arrangements: Spinal preparation. DLF, dissected dorsal part of lateral funicle at Th9. Dorsal column severed at Th10. VQ, dissected ventral quadrant at L1. H, hamstring nerve. Pairs of records show simultaneous discharge in left and right ventral quadrants (L. VQ and R.VQ). Upper records show unconditioned test discharges. Middle records show effect of repetitive stimulation of left DLF, and lower records effect of repetitive stimulation of right DLF. The right two vertical rows of records on slower time base. *Note*: Small early ipsilateral potential is due to activity in small part of dorsal spino-cerebellar tract included in the dissected ventral quadrant (cf. OSCARSSON 1956).

higher frequencies than those with small areas. In the decerebrate preparation in which the excitatory effects from the skin were strongly depressed, VSCT units were only exceptionally spontaneously active.

2. Suprasegmental control of tract neurones

Stimulation of the dorsal part of the lateral funicle (DLF) imitates the supraspinal control of interneurones (*cf.* HOLMQVIST *et al.* 1960). However, such stimulation also reveals a control system which can inhibit the VSCT neurones themselves. In Fig. 4 the hamstring nerve was stimulated and it was recorded from the dissected ventral quadrants as shown in the diagram. In the contralateral quadrant stimulation of the hamstring nerve evoked the monosynaptic

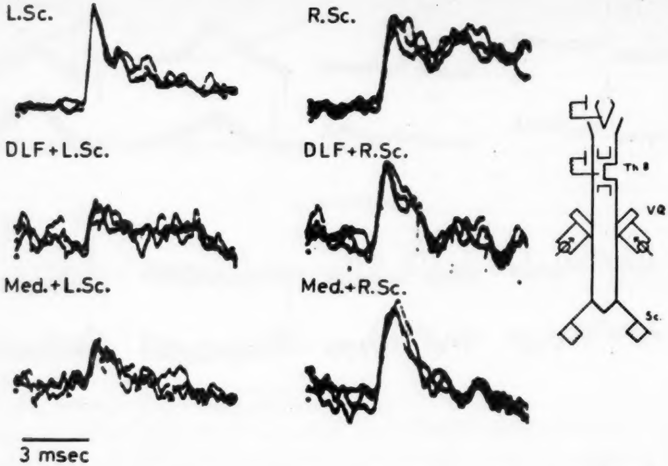

Fig. 5. Comparison of inhibition evoked from brain stem and from dorsal part of lateral funicle. The diagram shows stimulating and recording arrangements: Cerebellum removed. Needle electrode for stimulation of brain stem about 4 mm above obex. Surface electrode for stimulation of left dorsal part of lateral funicle (DLF) at upper Th8. Spinal cord transected on right side at lower Th8. Dorsal column severed at Th9. VQ, dissected ventral quadrant at L1. Sc, sciatic nerve. Upper records show test responses evoked from left and right sciatic nerves and recorded from right and left ventral quadrants respectively. Middle records: conditioning by repetitive stimulation of left DLF. Lower records: conditioning by repetitive stimulation of brain stem.

VSCT discharge due to the excitatory action from the Ib afferents. Repetitive stimulation of the DLF inhibited the VSCT response evoked from the nerve ipsilateral to the stimulated DLF. No effect was observed on the response from the contralateral nerve. The preparation was symmetric and for both sides the inhibition amounted to about 70 per cent of the unconditioned test value. The left two rows of records show that the inhibition of the late discharge is bilateral from each DLF as described by Holmqvist *et al.* (1960).

The inhibition of the VSCT neurones appeared at a strength of less than twice the threshold for activating fibres in the DLF. The effect became maximal at 6 to 8 times threshold. These values suggest that the velocity of the fastest fibres responsible for the inhibition was high but that also fibres of a fairly low velocity contributed. It was ascertained in special experiments that there was a good threshold separation between fibres of different velocities. The VSCT discharge evoked from the nerve contralateral to the stimulated DLF was uninfluenced even when the stimulus strength was increased to more than 10 times threshold.

Repetitive stimulation of the DLF initially evoked a discharge in certain fibres located in the ventral quadrant. Recording from single units showed that the inhibition of VSCT neurones was genuine and not due to refractoriness of

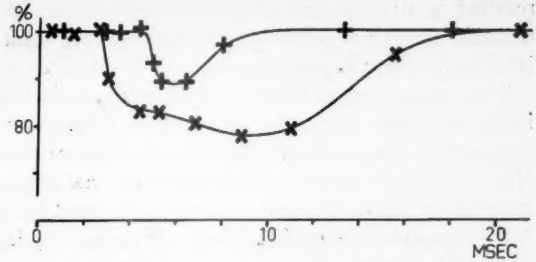

Fig. 6. Inhibition of VSCT neurones on single shock stimulation of brain stem and dorsal part of lateral funicle. Stimulating and recording arrangements as in Fig. 5 but with the hamstring nerve stimulated for testing. Ordinate: conditioned test response in per cent of unconditioned one. Abscissa: interval between conditioning and testing stimulus. x-markings: conditioning by stimulation of DLF. +-markings: conditioning by stimulation of brain stem. Distance between stimulating place in brain stem and spinal cord was 17.5 cm.

these neurones following activation. Repetitive stimulation of the DLF of either side never evoked any discharge in the VSCT units. Stimulation of the DLF ipsilateral to the nerve used for identification of VSCT neurones inhibited the resting discharge and the monosynaptic test response. At cessation of stimulation there was often a rebound showing up as a discharge of a few impulses.

Fig. 5 shows that also repetitive stimulation of the brain stem may inhibit the VSCT neurones. The sciatic nerves were stimulated and the VSCT discharges recorded from the dissected ventral quadrants. The right half of the spinal cord was sectioned at the Th8 segment. Repetitive stimulation of the left DLF inhibited the VSCT discharge from the left but not that from the right sciatic nerve. Similarly repetitive stimulation with a needle electrode in the lower brain stem inhibited the discharge from the left side but not that from the right side. In both cases the inhibition was about 60 per cent of the unconditioned test response.

The inhibitory effect could in some experiments be displayed after a single stimulus. The curves of Fig. 6 were obtained from an experiment with a similar stimulating and recording arrangement as that shown in Fig. 5. The VSCT discharge was inhibited when the conditioning stimulus to the DLF preceded the test stimulus to the hamstring nerve with about 3 msec. Stimulation of the brain stem resulted in a weaker inhibition with a latency of about 5 msec. The distance between the two stimulating places was 17.5 cm. Hence the fastest fibres responsible for the inhibition had a velocity of about 85 m/sec. The cells of origin of the VSCT fibres activated from the hamstring nerve are located in the L4 and L5 segments (Oscarsson 1957 b). The distance between these segments and the conditioning electrode of the DLF was approximately the same as the distance between these segments and the testing electrode of the hamstring nerve. The delay of about 3 msec makes it likely that more than one interneurone was interpolated between the fibres responsible for the inhibition and the VSCT neurones.

The inhibitory effect from the brain stem was weaker and of shorter duration than that from the Th8 segment (Fig. 6). Also repetitive stimulation often

resulted in stronger inhibition when applied to the spinal cord in the mid-thoracic region than when it was applied to the brain stem. Possibly part of the inhibitory system is propriospinal.

The control system suppressing transmission in the interneurones mediating inhibitory effects to the VSCT neurones is tonically active in the decerebrate preparation. In contrast, the system of fibres responsible for inhibition of the VSCT neurones was not tonically active in that preparation. The monosynaptic response of VSCT neurones was never observed to increase after spinalization. Neither was there any indication of a tonic activity in the spinal state in which most VSCT units have a resting activity.

Discussion

The group of neurones investigated constitute part of the ventral spino-cerebellar tract. These neurones have a complex organization of connections with primary afferents. They are monosynaptically excited by Golgi tendon organ afferents and in addition inhibited from group II and III muscle afferents, skin and high threshold joint afferents. Isometric contraction of muscles supplying a certain group of VSCT neurones with Ib excitation causes inhibition of these neurones in the spinal preparation. This is due to a dominance of the inhibitory effects from group II and III afferents over the monosynaptic excitation. Very strong effects are evoked from skin receptors in the spinal animal (which may be forwarded against the background of resting activity) and it has been suggested that the VSCT might be a tract conveying exteroceptive messages (Oscarsson 1957 a). Another suggestion is that the VSCT forwards information concerning flexion reflex patterns as it is influenced by afferents which may evoke the flexion reflex (Holmqvist *et al.* 1960).

It has recently been shown that the interneurones which transmit the inhibition from the 'flexion reflex afferents' (group II and III muscle afferents, skin and high threshold joint afferents; *cf.* Eccles and Lundberg 1960) to the VSCT neurones, are subject to a tonic inhibitory control in the decerebrate animal (Holmqvist *et al.* 1960). It has now been found that the existence of this inhibitory control profoundly alters the response of VSCT neurones to adequate stimulation of skin and muscle receptors. In the decerebrate preparation the effect evoked by adequate stimulation of the skin was very weak and from muscle Ib excitation was found to dominate over the inhibition from group II and III afferents. Apparently the tonic inhibitory control of the pathways from group II and III muscle afferents has brought out the Ib excitation which in spinal animals, because of the inhibitory effect from group II and III afferents never could be demonstrated in experiments with adequate stimulation of muscles (Oscarsson, 1957 a). Hence the nervous system is endowed with a mechanism which may change the message of the VSCT neurones from one which has been interpreted as exteroceptive or informative of flexion reflex patterns to one which is proprioceptive.

It is hardly probable that the VSCT neurones are utilized for conveying different types of messages at different times. This would necessitate a very complex mechanism for interpretation at the next synaptic relays. It seems more likely that the two types of information are integrated already when forwarded by the VSCT neurones. For instance, the main function of the inhibition from the flexion reflex afferents could be to cut off the effects from tendon organ afferents under certain conditions. The cerebellar cortex would still receive information from tendon organs via a subdivision of the dorsal spinocerebellar tract (LUNDBERG and OSCARSSON 1956) and this could serve as a reference for displaying the cut off effect in the VSCT.

A main difficulty in previous attempts to explain the significance of patterns of connections to VSCT neurones was the impossibility, in the spinal animal, to excite these neurones by Ib activation on adequate stimulation of the receptors. The present findings have relieved this difficulty and it is now of interest to discuss the type of message the tract neurones may forward because of their connections with Ib afferents. Two groups of VSCT neurones with different patterns of Ib connections have been described (OSCARSSON 1957 a). The neurones of one group were activated by Ib afferents from knee and foot extensor muscles (quadriceps, triceps). The neurones of the other group received Ib excitation from hip extensor, knee flexor, and foot extensor muscles (semimembranosus, anterior biceps; gracilis, semitendinosus, posterior biceps; triceps). These patterns of extensive convergence make it likely that the VSCT neurones convey information concerning posture or stages of movement rather than information of increased tension in single muscles.

If the tract forwards information based on the simultaneous inflow of impulses in Ib afferents from several muscles this would explain the fairly weak excitation obtained from a single muscle. Such a weak coupling would be a necessity if the neurones are to respond only to the combined increase of tension in several muscles and not to the isolated increase of tension in one muscle.

It has been suggested that the effects from group II and III muscle afferents, skin and high threshold joint afferents give information concerning flexion reflex patterns. The integrated message in the VSCT would then signal both posture or movement and stimuli evoking flexion reflexes. Such information would be of value for the cerebellum in regulation of compensatory movements in other limbs during the performance of a flexion reflex in one of the limbs. It is evident that a flexion reflex performed when the limb is supporting the body and when it is not supporting it requires different adjustments in the other extremities for keeping the balance.

Electrical stimulation of the dorsal part of the lateral funicle revealed a system which could inhibit the VSCT neurones. At least part of this system could be activated by stimulation of the brain stem. The responsible fibres were located in the same region as those responsible for the control of the interneurones mediating inhibition to the VSCT neurones. The new system

differed in that it was not tonically active in the decerebrate preparation and only affected ipsilateral VSCT neurones (the axons of which ascend in the contralateral ventral quadrant.)

The location of the cells of origin of these fibres has not been investigated. One possibility would be that they were ascending fibres with collaterals inhibiting the VSCT neurones. The fast ascending fibres in the dorsal part of the lateral funicle have been extensively investigated (LAPORTE *et al.* 1956 b, LUNDBERG and OSCARSSON 1959). Some of them are activated by the ipsilateral flexion reflex afferents which also strongly inhibit the ipsilateral VSCT neurones. This inhibition could possibly be mediated by collaterals of activated ascending fibres. However, the majority of these are spontaneously active whereas there has been no indication that the here investigated system is tonically active. Hence it is unlikely that this system is identical with any ascending tract. Probably the system consists of descending fibres. The cells of origin may be located in the brain stem or rostrally of the intercollicular section. One possibility to consider is the pyramidal tract which descends in the relevant part of the cord. On recording from lumbar segments of the spinal cord LLOYD (1941) observed inhibition of cells in the intermediate region of the grey matter following repetitive stimulation of pyramidal fibres. These cells, or some of them, were possibly identical with the cells of origin of the VSCT. The latter have recently been identified in the intermediate region and the ventral horn of lumbar segments (ECCLES, HUBBARD and OSCARSSON, unpublished).

Two systems have here been investigated which probably are used by higher centres for control of transmission to VSCT neurones. A third possible control system has been described previously. It consists of fast fibres located in the ventral quadrant with monosynaptic excitatory action on ipsilateral VSCT neurones (OSCARSSON 1957 a, p. 68).

Summary

1. Ventral spino-cerebellar tract (VSCT) neurones, monosynaptically activated by Golgi tendon organ (I b) afferents and with inhibitory action from group II and III muscle afferents, skin, and high threshold joint afferents have been investigated in unanaesthetized decerebrate cats.

2. In the decerebrate preparation the inhibitory actions are depressed due to a tonic inhibitory control of the interneurones mediating these actions (HOLMQVIST *et al.* 1960). It has been shown that this control profoundly alters the behaviour of the VSCT neurones.

3. In the decerebrate preparation isometric contraction of muscles with monosynaptic Ib connection to a certain group of VSCT neurones evokes excitation in these neurones. The inhibitory effects from group II and III afferents are depressed due to the tonic control. In the spinal state without this control the inhibitory effects dominate.

4. In the decerebrate preparation the effects evoked by adequate stimulation of cutaneous receptors are very weak. Strong pinching is needed and as a rule only inhibition is obtained. In the spinal state slight touch evokes strong inhibitory and excitatory effects from very wide receptive fields.

5. The functional significance of the various connections to VSCT neurones is discussed.

6. Repetitive stimulation of the dorsal part of the lateral funicle revealed a system of fibres which inhibits the VSCT neurones of the ipsilateral side. At least part of this system can be activated from the brain stem. It is not tonically active in the decerebrate preparation.

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On the Specificity of the Ruffini Like Joint Receptors

By

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Abstract

Eklund, G. and S. Skoglund. *On the specificity of the Ruffini like joint receptors.* Acta physiol. scand. 1960. *49*. 184—191. — The response pattern of Ruffini like receptors has been studied in some 60 knee joint capsule preparations, which in a Tyrode bath were exposed to a linear increase of tension. The response pattern was also studied while applying an additional tension increase at angles to the linear one. From the findings it was concluded that the sensitivity bands displayed by the sense organs in situ are only a reflection of tension alterations in the capsule and not of specific end organ properties.

The property of sensory receptors to respond more readily to one 'specific energy' than to others is called specificity (Müller's law). Many receptors in addition to this specificity, also display a specific sensitivity to a certain range of the adequate stimulus. This property, shown as an optimum sensitivity at a certain quality of the stimulus, is inherent in the sense organ. This is exemplified in the ear and the eye, while on the other hand the thermoreceptors (Zotterman 1953) show sensitivity optima at different temperatures, which is not a quality discrimination but still appears to be an inherent property of the sense organ, probably in respect to the disturbances of chemical equilibria set up by temperature changes (Granit 1955). As pointed out by Granit (1955) this is a characteristic solution of the problem of specificity whenever it is required to carry out important tasks of discrimination in a sense organ.

The joint capsule receptors of the Ruffini type (Boyd 1954, Skoglund 1956) also display optimal sensitivity with respect to angle of rotation when stimulated in situ by movements of the joint (Skoglund 1956). The problem is then, whether this discrimination reflects a specific property of the sense organ, or is brought about merely by variation of the stimulus which is tension.

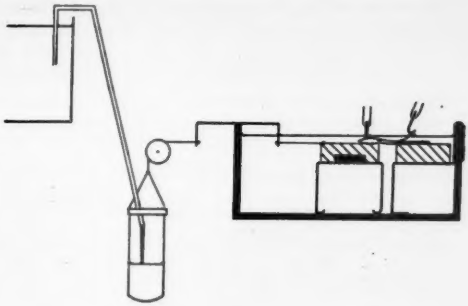

Fig. 1. Schematic drawing of the experimental arrangements. Heating device, pins and other details are excluded. The black field in the mobile piece of cork represents a lead weight heavy enough to prevent floating. For full description see text.

As long as the variations of tension in the capsule during movements are not known, the rather narrow sensitivity bands cannot be explained. By applying a stimulus controlled as far as is possible with respect to the receptors of a joint capsule, it might be possible to differentiate between a property of the sense organ and variation of the stimulus. It will here be shown that the latter is the probable cause of the narrow sensitivity bands.

Methods

Some 60 joint capsules from adult cats have been investigated. The cats were anesthetized with nembutal, chloralose or a combination of these, and the knee joint capsule and the medial nerve (GARDNER 1944) dissected out.

The dissection was started by opening the skin and detaching the insertion of the sartorius muscle at the medial aspect of the knee. After this the medial nerve was identified and carefully dissected free from surrounding connective tissue for 2—3 cm proximally down to its entrance into the capsule. Now the femoral artery was ligated, in order to avoid disturbing bleedings, while cutting out the preparation consisting of the patellar ligament including the patella, and the medial part of the joint capsule with the medial nerve. The medial collateral ligament was not included in the preparation. Then the capsule preparation was transferred into a bath, which contained about 4 l of fresh Tyrode's solution well oxygenated and kept at 33—37° C by means of 2 glass-insulated heater coils fed by 24 V. D. C., not altering the temperature more than 2° per hour. By means of pins the preparation was fastened to two pieces of cork. The patellar ligament was pinned to a mobile piece of cork and the medial, posterior part of the capsule to a fixed one. The preparation was thus horizontally suspended just beneath the surface of the Tyrode solution. The pulling string by which tension was applied to the preparation was connected horizontally to the mobile piece of cork. This was made possible by an aluminium sheet bent twice at right angles and thereby riding on the lid of the bath-box. The string was laid over a pulley and attached to a rubber container at the end. The weight of the container with its suspending ring was 10 g, which is about the same as the resting friction of the whole system. The thin walled container was capable of accepting practically unlimited amounts of water, which was delivered from a wide bottle via a siphon. This was done at a rate of 5 ml per sec. The height difference, that is the forcing power of the siphon, diminished by less than 0.7 per cent when delivering 100 ml of water. Thus rate of stimulation is kept constant. Fig. 1 is a schematic drawing of the experimental arrangements.

When recording, the nerve was lifted just above the surface of the Tyrode solution. The nerve impulses were led off by a silver electrode, fed to a push-pull amplifier through a cathode follower input, and made visible on a double-beam oscilloscope, with time on the spare beam. The impulses were also monitored by a loudspeaker. The reference electrode was mostly fastened to the patellar ligament, but at times hanging free in the Tyrode solution, which was grounded. Records were made on bromide paper.

Results

The capsule turned out to be a very resistant preparation independently of whether it had been taken out as fast as possible after starting the anesthesia, or if it was taken from cats used for other experiments lasting as long as 8 hours. Neither did the form of anesthesia used influence the sensory discharge notably. In some cases when the nerve was not dissected in order to 'isolate' fibres, the condition of the preparation was followed about 6 to 8 hours. During this time the sense organs did not deteriorate by any means, and even rapidly adapting organs were exhibiting normal behaviour after this time. It was also found that the preparation could be deprived of external oxygen supply for half an hour without notably affecting the impulses of an organ continuously firing during that time.

When slowly increasing the applied tension it could always be seen that more end organs were brought into play as the stimulation proceeded. In an undissected nerve it was never possible to record from single active organs up to their maximum frequencies without obtaining activity in other organs.

If the tension was increased rapidly the impulse frequencies showed overshoots. When, for example, a siphon delivering 25 ml/sec was used, the frequency quickly rose to 100—125 impulses/sec and then, in spite of further increase of tension, adaptation to maximum frequency normal for that end organ took place. An increase of 5 g/sec, however, did never produce such overshoots. Owing to the location of the end organs they will be activated at different external forces (Fig. 3), and consequently, there is no definite critical value at which the tension increase produces overshoots in all end organs.

The end organs were activated in different ways: stretching the capsule by means of a forceps in various directions and exerting pressure on the capsule with a glass rod. By these means it was not possible to detect any dissimilarities in the response of the organs; altering the tension always altered the impulse frequency in the same direction, independently of which type of stimulation was used and of its direction.

In most experiments it was possible to record from one or two functioning end organs after careful dissection of the nerve. When an 'isolated end organ' was obtained, it had to be decided where it was situated in the capsule. The reason for this was that tendon receptors had to be excluded because they do not have the same properties as Ruffini like endings, at least in situ. It is known that the tendon receptors are situated only in or around the ligaments of the

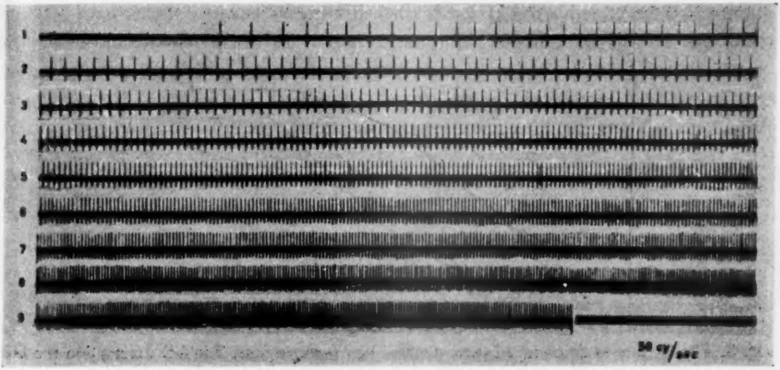

Fig. 2. The response of a single Ruffini like end organ to a tension increase of 5 g per sec. The strips are continuous. Time 50 c/sec. For full description see text.

knee joint (see Skoglund 1956). In our preparation the medial ligament was not included and so the tendon receptors there were probably excluded from the beginning. Those organs situated in the vicinity of the patellar ligament are not likely to be activated by a stimulus acting via the pulling string inserted at right angles to the ligament whose ends were fixed by pins. In trying to exclude, for certain, all tendon organs it was decided to use only those end organs which, when activated by probing the capsule with a glass rod, were found to be situated in the free part of the capsule between the patellar ligament and the site where the medial ligament was situated before it had been dissected away. When first trying to locate an active 'isolated end organ' it was however found that a slight pressure often caused alteration in the response of an organ from almost all parts of the capsule, and it was difficult to find a spot of maximum sensitivity. In further selecting only those end organs which responded to a slight pressure on the free part of the capsule but not to an even hard pressure in the vicinity of the location of the ligaments we find it highly probable that only Ruffini like end organs were used for the experiments.

In the records of Fig. 2 a typical experimental finding is illustrated. The records 1—9 are in direct continuation and each strip represents 4 sec as shown by the inset time (50 c/sec) corresponding to 1 sec. During each record the tension increased 20 g at a rate of 5 g/sec. Record 1 starts at 50 g reaching 55 g at the first impulse and 70 g at the end to continue from 70 g at the beginning of record 2 and reaching 90 g at its end and so on. An almost constant finding, the irregular onset, is seen during the first 10—15 impulses in record 1. The frequencies of this end organ are plotted as curve 'c' in Fig. 3.

In Fig. 3 the frequencies of three end organs from different experiments are plotted as ordinate against the tension applied to the whole capsule as abscissa. The load increases at a rate of 5 g/sec. The letters denoting each curve have

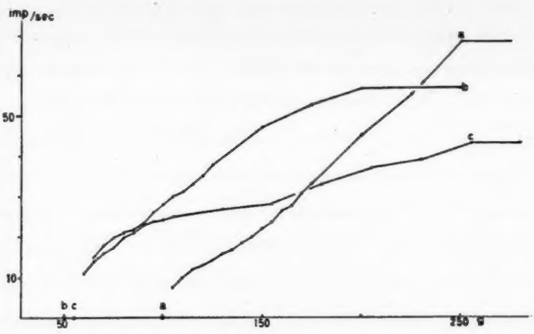

Fig. 3. The frequency response of three Ruffini like sense organs. For full description see text.

been drawn at the starting and top frequencies of each end organ. On account of the irregular onset the curves are not plotted during the first second, but then the frequency is calculated from counts over a whole second of continuous recording with standing spot. The curves which are plotted as far as the tension increase is going on, show clearly that the frequency of an end organ increases up to maximum value and then remains there in spite of further increase of tension. This was a constant finding from which must be concluded that an increase of tension in the capsule set up by a force in one direction does never produce anything looking like the sensitivity bands seen with these end organs in situ.

The end organs usually reached their maximum frequency at about a load of 200—300 g. With the method used it was often necessary to stop the tension increase at about 300 g to avoid destroying the preparation. This was usually, however, well above the load setting up the maximum frequency and so it is unlikely that any physiologically significant alterations took place after that. With maintained high tension above the value giving the maximum frequency for a particular end organ a slow adaptation usually took place, organ 'b' in Fig. 3 for instance adapted to 26 imp/sec after 30 min. Such a slow adaptation cannot be identical with or have anything to do with the sensitivity bands observed with these end organs in situ.

When the sense organs reached their maximum frequency the pulling string was unloaded to make sure that the impulses were set up by the tension invoked. This unloading procedure was performed because at times spontaneously firing end organs, as it seemed, were found. Such a spontaneous activity could always be abolished by moving one or two of the pins. Correspondingly end organs were found, which were very little affected by the load. In such cases the end organ appeared to be situated between two nearby pins.

Finally experiments were performed in which an additional tension was applied at angles to one already producing a relatively high or maximum fre-

quency in a single organ. When this was done the frequency was often unaltered, sometimes slightly increased, or temporarily decreased. In the latter case, however, it always regained its previous value, when the additional tension was further increased. In these experiments the frequency was never seen to diminish to zero. Thus, under such circumstances, no influence of the direction of the forces could be demonstrated to create the sensitivity bands seen with these end organs in situ.

Discussion

ANDREW (1954) in an investigation on the medial ligament found that the preparation survived without any marked deterioration for 12 hours in a bath of Locke's solution. He further noticed that no external oxygen supply, other than that provided by diffusion from the air, was necessary. The capsule preparations examined here were found to behave in conformity with these findings.

In the experiments where the capsule was probed with a glass rod, great difficulties were encountered to find the actual location of the sense organ. Since any alteration of the tension in the capsule in one point will effect remote points and probably do so even more in situ, it is obvious that the procedure of exploring 'innervation fields' by probing the capsule (STENER 1959) is of little validity.

From earlier investigations (BOYD and ROBERTS 1953, ANDREW and DODT 1953, SKOGLUND 1956) it is clear that the activation of the Ruffini like organs of the capsule is brought about by tension alterations. It is also obvious that the organs have properties in common with other mechano-receptors. In none of these works, however, has it been shown that the organs with increasing stimulation finally reach a maximum value, which is maintained throughout continuing tension increase. When increasing the pressure in the joint ANDREW and DODT (1953) found that "many large fibre sensory units were recruited and their individual frequencies increased". They do not mention any sustained top frequencies and no frequencies can be calculated from their picture as they do not give any time scale. Further, the concept of BOYD and ROBERTS (1953), that "It is probable, then, that the sense-endings are stretch receptors responding to extension in a particular direction", is not supported by our results. The ability of the Ruffini like endings to respond to tension whatever its direction, is a result in good accordance with their histological appearance (SKOGLUND 1956).

It is possible to assume that the property of the Ruffini like organs to respond to mechanical deformations in all directions might be the explanation of the sensitivity bands seen in situ. Added tension of another direction might cause a decrease of impulses, even to zero, by way of cathodal depression (KATZ 1950). It is interesting to note that the maximum frequencies may temporarily

diminish when additional tension is applied. However, the frequencies of the sensitivity bands obtained in situ seldom exceeded 40 imp/sec (SKOGLUND 1956), while those obtained from the capsule preparation exposed to a single linear tension as a rule are higher (cf. Fig. 3). This might be due to lack of additional tension, but a more attractive explanation is that the tensions in situ would not reach the magnitudes of those produced here.

With the method used in this investigation the tension in the whole preparation must, on an average, increase linearly. Despite this, the curves (Fig. 3.) almost always display 'steps'. These 'steps' can easily be explained by the somewhat non-uniform fibrous architecture of the capsule and also by the arrangement of the pins, which tends to deform the linear tension at the point of the sense organ.

From the steady increase of frequency to a maximum in response to increasing tension and from its final constant value or increase to further tension, it must be concluded that the sensitivity bands produced by movements of a joint are due to the way tension acts at the actual point where the end organ is situated, and not to any specific property of this organ. The decrease in some cases to additional tension is interesting, but this variation was only temporary and further increase of tension always reestablished the impulse frequency. This finding also makes cathodal depression an unlikely explanation of the sensitivity bands in situ. This form of specificity as it appears in the discharge of a sense organ in response to natural stimuli (joint movement) is thus only a reflection of the variations of tension. The possible requirements of such a form of specificity, as the sensitivity bands, for discrimination tasks in the central nervous system are open to speculations, but it is interesting to see that it is not in all cases created by the sense organ itself in any other sense than that it is acted upon by directional forces which increase or diminish tension at the point where the end organ is located.

Summary

The response of Ruffini like end organs in some 60 preparations from knee joint capsules have been studied. The preparations were held in an oxygenated Tyrode bath kept at physiological temperature.

1. The sense organs were activated by a linearly increasing tension applied to the capsule preparation by means of a siphon. The end organs were also activated by pressure and stretching of the capsule in different directions.

2. The frequency response obtained with the linearly increasing tensions show that the investigated sense organs behave like other slowly adapting mechano-receptors in general.

3. By applying additional tensions at right angles to the linearly increasing one when it produced a high or maximum frequency in a single organ, temporary alterations of frequencies could be produced, but the impulse discharge could never be silenced.

4. The experiments indicate that the sensitivity bands displayed by the investigated sense organs in situ are only a reflection of the tension variations in the capsule, *i. e.* variations of stimulus strength.

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A Colorimetric Method for the Determination of Calcium in Blood Serum

By

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Abstract

GRAN, F. C. *A colorimetric method for the determination of calcium in blood serum.* Acta physiol. scand. 1960. *49*. 192—197. — A colorimetric method is described allowing convenient and rapid estimation of 5—15 μg Ca in 0.1 ml of blood serum. N-hydroxy-naphtalene-1,8-dicarboxylic acid imide forms an insoluble salt with Ca, which is brought into solution by the addition of ethylene diamine tetra-acetic acid in excess. N-hydroxy-naphtalene-1,8-dicarboxylic acid imide has an absorption maximum at 338 mμ in alkaline solution; the optical density of the EDTA solution is therefore measured at this wavelength. Two moles of N-hydroxy-naphtalene-1,8-dicarboxylic acid imide will be equivalent to one mole of Ca. Tartaric acid, low concentrations of citric acid, phosphate, magnesium, zinc, and ferrous or ferric ions will not interfere in the determinations of Ca by this method. Higher concentrations of citric acid as well as the presence of manganese, strontium, and barium, are harmful and must be avoided.

The methods commonly employed are unsuitable for the estimation of very small amounts of Ca. Flame photometry may be used when special precautions are taken. Titrations with ethylene diamine tetra-acetic acid cannot be scaled down to the desired range. To this author's knowledge, the method described by NORDBÖ (1932) is the only procedure besides flame photometric methods

The following abbreviations have been used:
EDTA Disodium salt of Ethylene diamine tetra-acetic acid
Trisbuffer Tris(hydroxymethyl)aminomethane
Naphtalene imide N-hydroxy-naphtalene-1,8-dicarboxylic acid imide.
The distribution of the results is denoted by the standard error of the mean.

that will determine a Ca content of about ten micrograms in the sample with a sufficient degree of accuracy. Unfortunately, this method is tedious and time-consuming, and it requires considerable experience before good results are obtained.

Beck (1951) has found that sodium N-hydroxy-naphtalene-1,8-dicarboxylic acid imide will precipitate Ca quantitatively in an alkaline medium. The naphtalene imide ion is coloured and may be determined colorimetrically after the precipitate has been washed and brought into solution again in excess of EDTA, as shown by Amin (1957). In this laboratory we have been working on problems which have required analyses of Ca in very small volumes of blood serum. An investigation was therefore undertaken in order to extend the method of Amin (1957) to quantitative determination of five to ten micrograms Ca per sample; factors that possibly may interfere with this determination, have also been studied.

Methods

Reagents

A. 0.1 per cent sodium N-hydroxy-naphtalene-1,8-dicarboxylic acid imide, obtained from Fluka A. G. Chemische Fabrik, Buchs SG, Switzerland, under the name N,N-Naphtalyl-hydroxylamin natriumsalz.

B. 1 M ammonium chloride, 5 M ammonium hydroxide buffer.

C. 0.1 M EDTA solution.

D. Washing solution prepared by diluting reagent B ten times.

E. A solution made by mixing one part of reagent B and four parts of reagent C.

F. Standard Ca solution is made from dried calcium carbonate by dissolving the salt in a small excess of hydrochloric acid.

All the solutions are made from reagent grade chemicals and distilled water. The naphtalene imide was obtained as a purified preparation of sufficient purity for this purpose.

Procedure

A suitable aliquote containing from 5 to 15 μg Ca is pipetted into a 15 ml conical centrifuge tube. Water is added to make a volume of 2 ml, followed by the additions of 1 ml of reagent B and 1 ml of reagent A in the order given.

The precipitation is complete after 3 hours at room temperature. The tubes are centrifuged at 1,000 $\times$ g for 10 min. The supernatant is carefully removed by gentle suction through a capillary tube or by very careful decantation. Ten ml of reagent D is added, the precipitate is suspended, and next centrifuged. The supernatants are discarded.

Five ml of reagent E is added to each tube, the tubes are placed in a rack on a boiling water bath until the precipitate is completely dissolved. The content is transferred quantitatively to a volumetric flask of suitable volume (25 ml) and the flask is made up to volume with reagent E.

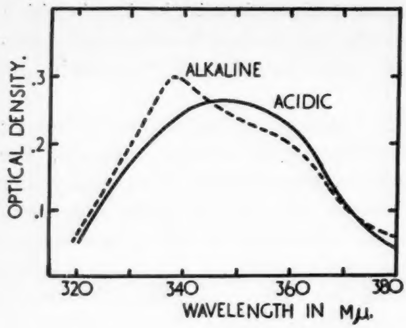

Fig. 1. Spectra for N-hydroxy-naphtalene-1,8-dicarboxylic acid imide in alkaline and acidic medium.
The spectra were taken in a Beckman DU spectrophotometer using 1 cm long cells. The concentration corresponds to 0.4 μg Ca per ml final solution. The spectrum in acidic medium was taken in 0.1 N hydrochloric acid, whereas the spectrum in alkaline solution was taken in the EDTA-ammonia buffer routinely employed.

The optical density is measured in a Beckman DU spectrophotometer at 338 mμ against a blank of reagent E, using 1 cm absorption cells of the square type.

Results

The absorption spectra for naphtalene imide were determined in alkaline and acidic solutions, see Fig. 1. The spectra were found to differ somewhat in the two media; in alkaline solution the absorption maximum was found at 338 mμ, whereas there was a change to 346 mμ when the spectrum was taken in 0.1 N hydrochloric acid.

Amin (1957) has reported that the maximum was at 400 mμ. The reason for this divergence is not known.

The molar extinction coefficients in different solutions have been calculated and are reported in Table I.

Table I. The molar extinction coefficients for Ca-N-hydroxy-1,8-dicarboxylic acid imide

Medium	Wavelength	E
Alkaline	338 mμ	2.96×10^4
Alkaline	400 »	3.46×10^3
Acidic	338 »	2.35×10^4
Acidic	346 »	2.66×10^4

The molar extinction coefficients have been obtained from measurements of the optical densities in a Beckman DU spectrophotometer. The final concentrations corresponded to 0.4 μg Ca per ml. One cm long absorption cells were used. The molar extinction coefficients for the naphtalene imide is one half of the values given.

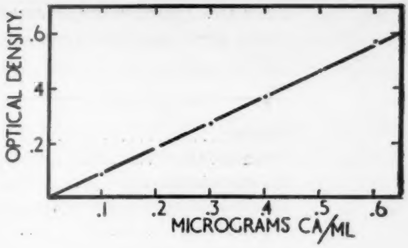

Fig. 2. Standard curve for Ca-N-hydroxy-naphtalene-1,8-dicarboxylic acid imide. The optical densities were measured in a Beckman DU spectrophotometer at 338 mμ, using 1 cm long cells. Various amounts of Ca was precipitated according to the procedure described in the text. Final volume was 25 ml, giving amounts of Ca per ml as indicated on the abscissa.

A typical standard curve is given in Fig. 2. The curve has been prepared by precipitating different amounts of Ca according to the procedure previously described. The light absorption follows Beer's law up to a final concentration equal to 0.7 μg Ca per ml.

Table II. The precipitation of Ca in the presence of various substances

Additions to sample			Micrograms Ca recovered
None			10.0 ± 0.01
0.1 M trisbuffer, pH	9.0		9.9 ± 0.02
Magnesium sulfate	0.05	mg Mg	$9{\cdot}8 \pm 0.02$
» »	0.5	mg Mg	9.8 ± 0.02
Barium chloride	0.01	mg Ba	13.3 ± 0.04
Strontium chloride	0.01	mg Sr	13.2 ± 0.03
Manganese sulfate	0.01	mg Mn	16.0 ± 0.04
Zinc sulfate	0.01	mg Zn	10.0 ± 0.02
» »	0.05	mg Zn	11.1 ± 0.03
Ferrous sulfate	0.01	mg Fe	10.0 ± 0.03
» »	0.05	mg Fe	10.4 ± 0.06
Ferric suifate	0.01	mg Fe	10.1 ± 0.02
» »	0.05	mg Fe	11.2 ± 0.05
Citric acid	0.2	mg	9.9 ± 0.02
» »	2.0	mg	9.9 ± 0.04
» »	5.0	mg	7.9 ± 0.03
» »	10.0	mg	0.4 ± 0.02
Tartaric acid	2.0	mg	9.9 ± 0.02
Potassium phosphate	0.5	mg P	9.8 ± 0.03

The values are means $\pm$ standard error of the mean.

The additions were made as indicated above to 10 μg Ca, the sample was diluted to 2 ml with water followed by the addition 1 ml ammonia buffer (in one case replaced by tris buffer), and 1 ml 0.1 per cent naphtalene imide. The tubes were left at room temperature for 3 hours. The optical density was measured in a Beckman DU spectrophotometer at 338 mμ, after the precipitate had been washed and dissolved in EDTA-ammonia buffer, and diluted to 25 ml. Eight samples were used in each series of determinations.

Table III. Typical values for serum Ca in rats

Group	Number of rats	Serum-Ca mg %
Normal	16	10.2 ± 0.1
Vitamin D free	16	7.2 ± 0.2
Parathyroidectomized	16	5.8 ± 0.2
Parathyroidectomized and vitamin D free	16	4.8 ± 0.1

Mean values ± standard error of the mean.

0.1 ml serum was diluted to 2 ml with distilled water, 1 ml ammonia buffer was added followed by 1 ml 0.1 per cent naphtalene imide and left at room temperature for 3 hours. The optical density was measured in a Beckman DU spectrophotometer at 338 mμ after dissolving and diluting the precipitate to 25 ml with EDTA.

The stoichiometry of the precipitate was found to be one atom of Ca per two molecules naphtalene imide.

In Table II are reported recoveries obtained when the precipitation of 10 μg Ca had been carried out in the presence of different ions which might interfere with the determinations. Eight samples were prepared in each series.

Additions of up to 500 μg Mg per sample did not affect the determination of Ca. Strontium, barium, and manganese added in amounts equal to the amount of Ca present in the sample, were partially precipitated and will hence disturb the determinations. When higher levels of these ions are present, the error will increase. The presence of 10 μg of zinc, ferrous or ferric ions, did not cause any errors, however, these ions will be partially precipitated when the level is increased to 50 μg.

The addition of 2 mg tartaric acid or 0.5 mg phosphorus added as potassium phosphate, did not have any effect. Citric acid, which is known to form stable complexes with Ca, prevents the complete precipitation of Ca if more than 2 mg is present in the sample.

The determinations may be carried out in 0.1 M Tris buffer at pH 9.0 instead of the ammonium chlorideammonium hydroxide buffer usually employed.

The recovery of known amounts of Ca added to blood serum has been found to be complete. A typical experiment will be reported here. A pooled batch of serum taken from parathyroidectomized rats, was analysed for Ca according to the methods of Nordbö (1932), Wang (1935), and the method described, and was found to contain 5.2 mg Ca per 100 ml. Eleven aliquotes of 0.05 ml each were taken from this batch, 10 μg Ca was added to each tube. The analyses were carried out as previously described. The mean value was exactly the one to be expected: 12.6 ± 0.2 micrograms Ca per sample.

In Table III are listed typical values observed for Ca in blood serum taken from young rats, and obtained by the method reported. The data were found to be in excellent agreement with figures obtained by the method of WANG (1935), and NORDBÖ (1932).

The Ca salt of naphtalene imide was found to be insoluble in organic solvents such as ethyl alcohol, isopropanol, butanol, chloroform, benzene, light petrol ether, ether, and trichlorethylene; no increase in absorbancy at 338 mμ could be observed in any of these solvents after they had been equilibrated for one hour with the salt at room temperature.

In a personal communication professor Eeg-Larsen of the Faculty of Dental Medicine at this University, reports that the method was readily brought into work and without any difficulties. In his laboratory the method was scaled down for the quantitative estimation of approximately 1 μg Ca in histochemical work.

The author is indebted to Miss GERD THUNE for valuable analytical assistance.

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Dynamic Elasticity in the Initial Phase of an Isotonic Twitch

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Abstract

Buchthal, F. and P. Rosenfalck. *Dynamic elasticity in the initial phase of an isotonic twitch.* Acta physiol. scand. 1960. *49*. 198—210. — A method is described which allows a continuous determination of the elastic and viscous component of the dynamic stiffness of isolated frog muscle fibres in vibration experiments. Elastic and viscous stiffness increased gradually in the initial phase of preloaded isotonic twitches. The increase started already within the mechanical latent period. Within 25 per cent of the time to maximum shortening elastic stiffness increased by 100 per cent and viscous stiffness by 50 per cent. In slightly stretched fibres, at the length in the body, the increase in dynamic stiffness during tetanic contraction was identical with that found in a twitch. In highly stretched fibres dynamic stiffness in a tetanus exceeded that of a twitch, presumably due to the redistribution of load between the contractile substance and the sarcolemma. The increase in dynamic stiffness during a twitch immediately after a prolonged tetanus was markedly lower than in a twitch performed before the tetanus. The contributions to the dynamic stiffness from the parallel elastic, the series elastic and the contractile component were evaluated. Although the periodical deformations were essentially confined to the series elastic component, the dynamic elastic properties give information about the contractile substance as the series elastic component mainly represents the elasticity of the contractile material.

Many investigations have been made in recent years with the object of correlating the time course of the mechanical, electrical, optical and chemical events occurring in striated muscle during a twitch contraction. Several types of experiments indicate that the structural changes initiated by the stimulus start before the development of tension or the shortening: measurements of

the latency relaxation (SANDOW 1944, ABBOTT and RITCHIE 1951 a), transparency and diffraction (D. K. HILL 1949, 1953), heat production (HILL 1949 a, 1950 a, 1953 b), extensibility (BUCHTHAL and KAISER 1944, HILL 1950 b, 1951 b) and torsional elasticity (STEN-KNUDSEN 1953).

As regards the elastic properties REICHEL and BLEICHERT (1955) using alternating changes in length of 1—4 per cent did not find any change during the mechanical latent period of isometrically contracting tortoise muscle, while HILL (1950 b, 1951 b) with quick stretches of about 10 per cent of the muscle length found a significant decrease in the extensibility of tortoise and frog muscle before tension was developed. In this investigation we have studied the changes in dynamic elastic properties of frog muscle fibres which occur in the initial phase of an isotonic twitch[1].

On the basis of his three-component model of muscle HILL (1953 a) considers "mechanical methods alone inconclusive in the study of the elastic properties of active muscle since their results are largely, probably mainly, due to the inert elastic elements from which the contractile component cannot be isolated". To test the justification of this objection the findings of this study were analysed in terms of this model.

Methods

a) *Definition of elastic and viscous stiffness:*

If a muscle fibre which is stretched by an external load is subjected to a periodically alternating force, simultaneous recording of force and movement reveals a phase displacement, the change in length lagging behind the changes in load. The change in length $\gamma(t)$ produced by a sinusoidally alternating force $\sigma_o \cos \omega t$ of amplitude σ_o and angular frequency ω, is nearly sinusoidal:

$$\gamma(t) = \gamma_o \cos (\omega t - \psi) \quad \ldots\ldots\ldots\ldots \quad (1)$$

where γ_0 is the length amplitude and ψ the phase difference between force and length. Hence, the movement can with good approximation be described by an equation of motion of the type:

$$m \frac{d^2\gamma}{dt^2} + \eta \frac{d\gamma}{dt} + G_{elast}\, \gamma = \sigma_o \cos \omega t \quad \ldots\ldots\ldots\ldots \quad (2)$$

where m is the equivalent mass of the oscillating system. The visco-elastic properties of the muscle fibres may hence be characterized by the *elastic stiffness* G_{elast} and the *viscous stiffness* $G_{visc} = \eta\omega$, where η is the damping as defined by (2) (BUCHTHAL, KAISER and ROSENFALCK 1951). This description of the visco-elastic properties of the muscle in terms of a damped elasticity must not, however, be taken too literally. It turns out that elastic and viscous stiffness depend on the frequency and amplitude of the vibrations (BUCHTHAL, KAISER and ROSENFALCK 1951).

Inserting equation (1) into (2) the following expressions for G_{elast} and G_{visc} are obtained:

$$G_{elast} = \frac{\sigma_o}{\gamma_o} \cos \psi + m\omega^2 \quad \ldots\ldots\ldots\ldots \quad (3)$$

$$G_{visc} = \frac{\sigma_o}{\gamma_o} \sin \psi$$

[1] Preliminary report: BUCHTHAL and ROSENFALCK (1953).

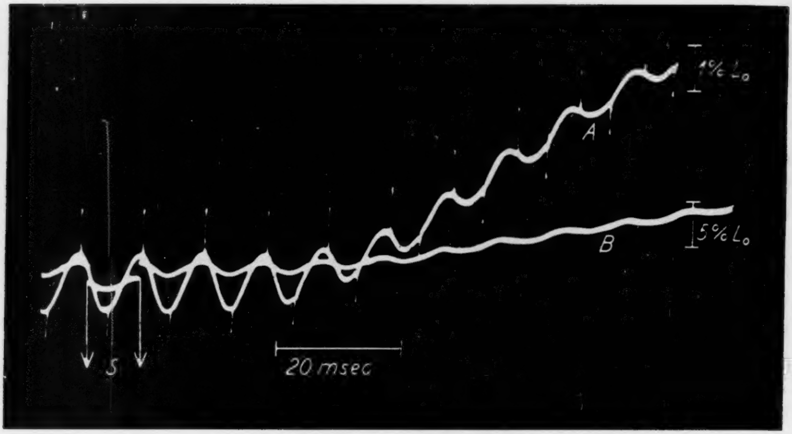

Fig. 1. The course of shortening in an isotonic twitch with superimposed vibrations recorded simultaneously with high (A) and low (B) amplification. The fibres were stretched to length 1.7 L_0. The vibrational frequency was 100 cps and the initial amplitude 0.7 per cent of L_0 (0° C).
The moments at which the vibrational force was zero are indicated by the vertical peaks superimposed on the alternating changes in length (A). The stimulus mark (S) was recorded on B.

Previously (Buchthal, Kaiser and Rosenfalck 1951) we have determined these parameters under stationary conditions, *i. e.* in resting or in tetanically contracted fibres. Elastic and viscous stiffness were measured at resonance frequency *i. e.* the frequency at which $\psi = \frac{\pi}{2}$. In the present study the phase difference ψ was measured directly and G_{elast} and G_{visc} could be determined at any desired frequency and under non-stationary conditions. It is clear from equations (3) that a determination of elastic and viscous stiffness requires measurement of the length amplitude γ_0 and the phase difference ψ. Furthermore, the force amplitude σ_0, the frequency ω of the alternating force and the equivalent mass m of the oscillating system have to be specified.

Experimental technique

The aim of the present study was to determine the time course of the changes in elastic and viscous stiffness in the initial phase of a twitch contraction of a small bundle of muscle fibres.

Since the mechanical properties of the contractile substance depend on the load it was desirable to use a type of contraction during which the load remained constant. Even during an isotonic twitch the load on the contractile substance may increase when the fraction of the load carried by the sarcolemma decreases with shortening. However, in the initial phase of an isotonic twitch the shortening was so small that even in highly stretched fibres the load carried by the contractile substance did not change to a degree which influenced dynamic elasticity. On the other hand, the redistribution of load between contractile substance and sarcolemma was appreciable when the tetanic level was reached in stretched fibres (cf. p. 205).

Therefore isotonic twitches were studied and in most experiments the muscle fibres

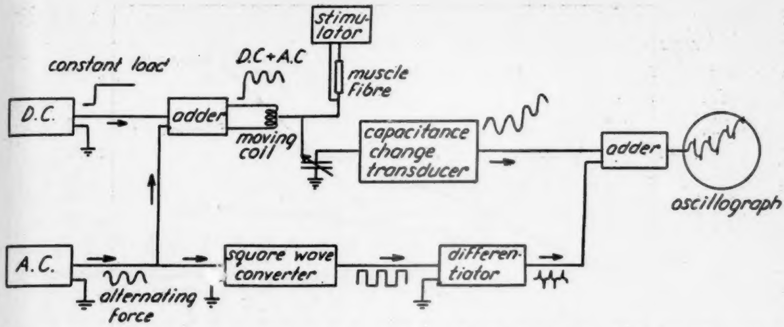

Fig. 2. Block diagram of the set-up used to indicate the moments at which the alternating force is zero. The constant and alternating loads were applied to the muscle fibres via the moving coil of the condenser myograph. The markings were obtained by converting the sinusoidal voltage to square waves and then differentiating them.

were stretched well beyond their body length by a load amounting to one third to half the maximum tetanic force in order that the alternating changes in load should be small as compared with the constant load. At resting loads of this magnitude the sarcolemma contributes substantially to the stiffness measured (CASELLA 1951).

In some experiments where twitch and tetanus were compared the fibres were only stretched to about their length in body so that the sarcolemma did not influence the measurements.

Bundles containing 4—5 fibres from the frog's m. semitendinosus were mounted in the isotonic myograph previously described (BUCHTHAL, KAISER and ROSENFALCK 1951). The constant load on the fibres and the superimposed alternating force were obtained by passing a constant and an alternating current through the coil of the myograph. The movements of the fibre bundle arising from the active shortening and from the alternating load were transformed into voltage changes via the changes in capacity of a condenser, one plate of which was fixed and the other attached to the moving coil. The sensitivity for changes in length was 154 mV per mm. The alternating force was chosen so that it gave a length amplitude γ_o at rest of 0.7—0.8 per cent of the equilibrium length L_o of the fibre bundle. The corresponding amplitude σ_o of the alternating load was 60—80 dynes which was produced by a current of 6—8 mA through the coil. The frequency of vibration varied between 60 and 100 cps.

The oscillating system had an equivalent mass of 15 mg. In Ringer's solution the system itself had an elastic and a viscous stiffness of about 150 dynes/cm, negligible as compared with those of the fibres of 5,000—15,000 dynes/cm.

The changes in fibre length, viz. the changes in capacity were recorded on a double-beam oscilloscope, with one beam to resolve the initial course of shortening, and the other beam to record the entire course of the twitch or the tetanus (Fig. 1). The maximum shortening velocity during the twitch contraction was 0.2—0.75 L_o per sec. and the maximum shortening amounted to 6—24 per cent of the equilibrium length L_o of the fibres. L_0 is defined as the length at load 0.005 P_o, P_o being the maximum load which the fibres can carry without shortening in a preloaded isotonic contraction. L_o varied between 12 and 15 mm. To secure simultaneous stimulation of all parts of the fibre bundle stimulation was performed by square wave pulses via a multi-electrode assembly covering the entire length of the bundle.

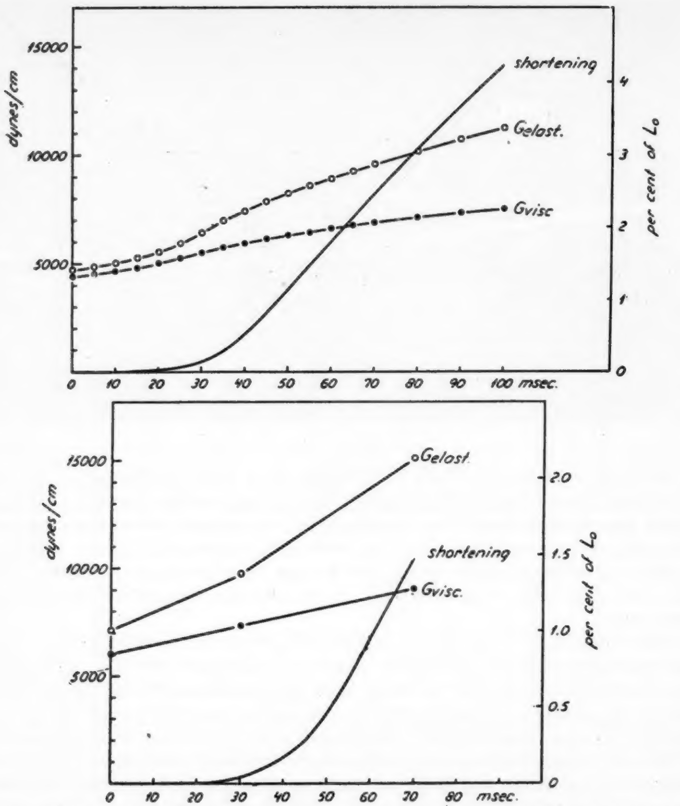

Fig. 3. Elastic stiffness (G_{elast}), viscous stiffness (G_{visc}) and shortening during the initial phase of an isotonic twitch (load $= 0.3P_0$; initial length 1.8 L_0). In *a* the vibrational frequency was 100 cps, in *b* 60 cps. The initial amplitude was 0.8 per cent of L_0. The maximum shortening of 18 per cent in *a* and 12 per cent in *b* was attained within 400 and 500 msec, respectively (0° C). Left ordinate: stiffness in dynes/cm. Right ordinate: shortening in per cent of L_0. Abscissa: Time after stimulus in msec.

A marking indicating the moments of the zeros of the alternating force was introduced on the beam of the oscilloscope recording the initial course of shortening at high amplification and was used for the determination of the phase difference between force and movement. For this purpose the sinusoidal force was fed to an overloaded amplifier and recorded as square waves. These were differentiated to appear as short spikes of alternating sign (Fig. 2).

The length amplitude γ_0 and the phase difference ψ were measured from the envelope curves and the mean course of shortening on enlarged photographs.

The experiments were performed at 0° C.

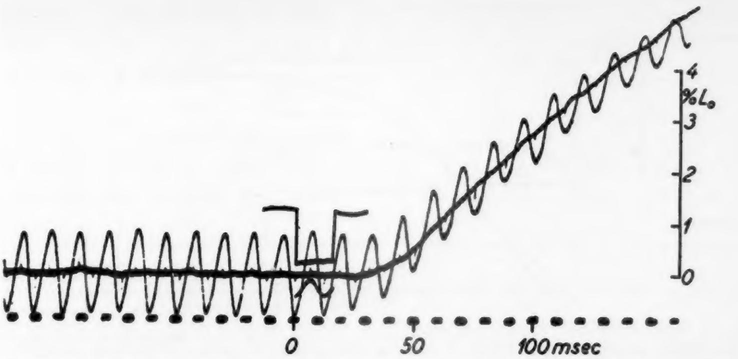

Fig. 4. To show that the longitudinal vibrations did not activate the contractile material of the muscle fibres. A twitch without vibrations is shown superimposed on a twitch with alternating changes in length. Load 0.4 P_0; initial amplitude = 0.75 per cent of L_0; vibrational frequency = 80 cps. The stimulus is indicated by the rectangular pulse at 0.

Results

A. *The change in stiffness in the initial phase of an isotonic twitch*

During the development of an isotonic twitch elastic and viscous stiffness increased gradually within more than 15—25 per cent of the time to maximum shortening (Fig. 3 a and b). An increase in stiffness could be recognized already at the end of the mechanical latent period. Before a measurable shortening had occurred, 15 msec after the stimulus, elastic and viscous stiffness had increased by 10 per cent (Fig. 3 a). When the shortening velocity had reached its maximum, 70 msec after the stimulus, elastic stiffness had increased by 100 per cent and the viscous stiffness by 50 per cent. At this time the fibres had shortened by only 2.4 (Fig. 3 a) and 1.5 (Fig. 3 b) per cent of L_o. The maximum shortening, 18 and 12 per cent of L_o, was obtained within 400 and 500 msec.

The increase in stiffness had reached its maximum 250 msec after the stimulus. The course of shortening was only slightly affected by the superimposed vibrations. The latent period was about 20 per cent shorter with than without vibrations, and shortening with was 3—10 per cent less than without vibrations (Fig. 4).

In fatigued muscle fibres viscous stiffness remained practically unchanged throughout the twitch, while elastic stiffness started increasing already during the latent period (Fig. 5). At the end of this period, *i. e.* 60 msec after the stimulus, the elastic stiffness exceeded its resting value by 30 per cent. During shortening elastic stiffness rose further to a maximum of 50 per cent at the peak of shortening at 700 msec.

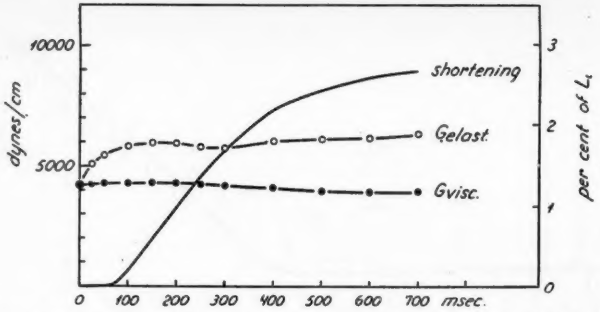

Fig. 5. Fatigued fibres. Elastic stiffness (G_{elast}), viscous stiffness (G_{visc}) and shortening during an isotonic twitch. Initial length = 1.5 L_0. Vibrational frequency = 60 cps. Initial amplitude = 0.75 per cent of L_0 (0° C).

B. *Stiffness in a twitch and in a tetanus*

The finding of gradual increase in stiffness in the initial phase of an isotonic twitch made it desirable to investigate whether a further increase in stiffness occurred in a tetanus. Experiments were performed at 10—30 per cent of stretch to avoid the influence of the parallel elasticity. The elastic and viscous stiffness were at the tetanic level equal to the maximum stiffness observed during the isotonic twitch.

C. *Effect of a preceding, prolonged tetanus*

Shortening was delayed in a twitch initiated immediately after a prolonged tetanus (about 5 sec in duration) the shortening velocity being half that before the tetanus. Also the changes in stiffness in the initial shortening phase were reduced (Table 1). Seventy msec after the stimulus, elastic and viscous stiffness had increased by 45 and 40 per cent in the twitch initiated after the tetanus whereas the increase in a twitch before the tetanus were 110 and 50 per cent.

Discussion

The main result of the study presented in this report was that the decrease in the extensibility of a small bundle of frog muscle fibres in a preloaded isotonic twitch began within the latent period and continued for at least 100 msec (0° C), *i. e.* for about 20 per cent of the time to peak shortening. The early onset of the increase in stiffness is in agreement with previous findings in quick stretch experiments in isometric (Hill 1950 b, 1951 b) and in isotonic contractions (Buchthal, Kaiser and Rosenfalck 1951) and with vibrational experiments in isometric contractions (Buchthal and Kaiser 1944). Similarly, the rise in torsional stiffness begins within the latent period of the isometric twitch contraction (Sten-Knudsen 1953). Reichel and Bleichert (1955)

Table I. Effect of preceding tetanus on stiffness changes during a twitch

time after stimulus (msec)	twitch before tetanus				twitch after tetanus			
	0	30	70	540	0	30	70	630
Elastic stiffness (dynes/cm)	7,000	10,000	14,750	—	7,200	7,450	10,550	—
Viscous stiffness (dynes/cm)	6,250	7,700	9,450	—	6,200	6,800	8,800	—
Shortening (per cent of L_0)	0	0.05	1.67	12.5 (max.)	0	0.02	0.39	11.7 (max.)

failed to observe an increase in vibrational stiffness during the latent period of an isometric twitch.

It might be argued that the early increase in stiffness was due to an activation of the contractile substance by the mechanical deformations (PRINGLE 1949). In fact, the 20 per cent decrease in latent period for twitches with superimposed vibrations might be interpreted as an activation while the slightly smaller shortening with than without vibrations points against this interpretation.

As to the time course of the increase in stiffness during the twitch one might question whether the change in the visco-elastic properties in fact is gradual since an instantaneous change might appear as a gradual change because of the delaying viscosity. This "gradual" change in stiffness would, however, be confined to a fraction of a cycle of the superimposed alternating force[1], and the observed gradual increase in stiffness was not an artifact.

On the other hand, the changes in stiffness were confined to that phase of contraction in which twitch and tetanus coincide, the increase in stiffness during an isotonic tetanic contraction being identical with that found in a twitch. This finding refers to experiments in which the fibre bundle was stretched to about its length in the body. Thereby the influence of the decrease in length of the sarcolemma during shortening was avoided. That the decrease in length of the sarcolemma may play a role at the tetanic level was indicated by the finding of a higher stiffness during a tetanus than during a twitch in highly stretched fibres (BUCHTHAL and ROSENFALCK 1953). This difference is probably due to the redistribution of load between the sarcolemma and the contractile substance as shortening proceeds. When the tetanic level is attained there are signs of a further structural reorganization ("elastic looking", BUCHTHAL, 1942, BUCHTHAL, KAISER and ROSENFALCK 1951; slow stretches and releases, ABBOTT and AUBERT 1952).

As to the interpretation of the results obtained in vibration experiments HILL (1953 a) has raised a number of objections implying that this type of

[1] This has been shown by calculating the length changes in a vibrating Kelvin element whose elasticity and viscosity were suddenly increased. With the values for these parameters of resting and contracted muscle fibres the time constant of adjustment was less than one fourth of the period of oscillation.

experiments can hardly give information about the contractile material. These objections, if valid, concern all previous studies of muscle elasticity including those using quick stretch (HILL 1949 b, 1950 b, 1951 b) and it seemed of interest to evaluate their relevance. According to HILL (1949 b) the mechanical properties of muscle are conveniently described by a model consisting of three components: a) a contractile component, b) an undamped series elasticity and c) a parallel elasticity. In the following the extent is determined to which these different components contribute to the elastic and viscous stiffness as measured *e. g.* in the experiments presented in this report. In this connection attention will be drawn to the fact that the contractile and series elastic components are defined by HILL in an operational way and were not given separate structural correlates as has recently been discussed in detail by PRINGLE (1960).

(1) HILL (1953 a) objects that unless the length of the muscle is small enough the parallel elastic component also is involved.

The parallel elastic component of frog muscle fibres is considered to be localized mainly in the sarcolemma (HILL 1949 b). Originally RAMSEY and STREET (1940) found that the length-tension curve of the intact resting fibre coincided with that of its empty sarcolemma tube. A reinvestigation of this problem showed that only at loads exceeding 0.2 P_0 a significant fraction of the load was carried by the sarcolemma (CASELLA 1951). At the load applied in most of our experiments (0.5 P_0) about half of it was carried by the sarcolemma (0.3 P_0). As measured with longitudinal vibrations the dynamic stiffness of fibres with partly empty sarcolemma tubes was smaller than that of the intact fibres (STEN-KNUDSEN 1951) and the stiffness-load relationship indicates that in our experiments the sarcolemma contributed about 50 per cent to the dynamic stiffness of the resting fibres.

HILL (1953 a) furthermore objects that the non-linearity of the stress-strain relation of the parallel elasticity introduces an important complication. However, with the small deformations applied in vibrational experiments (about 1 per cent) the non-linearity was without influence.

(2) HILL (1953 a) objects that unless the frequency is high, finite physiological shortening will occur during each phase of falling tension, and the non-linearity of the characteristic relation between velocity of shortening and tension again introduces a complication.

We have taken active ("physiological") shortening into account by measuring the length amplitude of the vibrations as the distance between the envelope curves during shortening (cf.p. 202). On account of the dependence of the shortening velocity on the load the alternating force caused varying shortening velocities. This affected the amplitude of the resulting length oscillations in the following way:

The active shortening of the contractile component of fully active muscle is described by HILL's (1938) equation:

$$V = \frac{P_o - P}{P + a} \cdot b, \qquad (4)$$

where V is the shortening velocity, P the load on the contractile component, P_0 the tetanic force and a and b are constants. A small alternating change in load $\Delta P = \sigma_0 \cos \omega t$ brings about an alternating change in shortening velocity

$$\Delta V = -\frac{(P_o + a) \cdot b}{(P + a)^2} \cdot \sigma_o \cos\omega t \qquad (5)$$

which in turn gives rise to an alternating length change $\gamma(t)$ of the fibres of:

$$\gamma(t) = \frac{(P_o + a)b}{\omega(P + a)^2}\, \sigma_o \sin\omega t \qquad (6)$$

This corresponds to the change in length of a viscous element with damping $\eta_c = (P + a)^2 \;/\; (P_o + a)b$ and hence stiffness

$$G_c = \frac{(P + a)^2\omega}{(P_o + a)b} \qquad (7)$$

when subjected to the alternating load σ_o cos ωt. Hence, the contractile component of fully active muscle behaves in vibration experiments as this viscous stiffness. With a load on the contractile component of 0.2 P_o and a vibrational frequency of 60 c.p.s. this stiffness of the contractile component is calculated to be 136 $P_o L_o^{-1}$($a/P_p = 0.25$ and $b = 0.45\ L_o$/sec, HILL 1938). This is about ten times the total dynamic stiffness ($= \sqrt{G^2_{visc} + G^2_{elast}}$) of the resting fibres (11—13 $P_o L_o^{-1}$, this study and BUCHTHAL, KAISER and ROSENFALCK 1951). Therefore, since furthermore a considerable part of the stiffness of the fibres was attributed to the parallel elastic component, the length changes of the contractile component arising from the variations in shortening velocity amounted to less than ten per cent of the observed vibrational amplitude.

It is, however, not justified to conclude from this high stiffness of the contractile *component* deduced from the force-velocity relation that vibration experiments cannot give information about the contractile *substance* (cf. p. 208).

(3) HILL (1953 a) states that physiological shortening during the phase of falling tension requires (if the average length is to remain constant) a corresponding lengthening during the rising phase. There is, however, a discontinuity in the characteristic relation at zero speed, — $\Delta V/\Delta P$ for a small increment of tension above full isometric level being considerably less than $\Delta V/\Delta P$ — for a small decrement of tension from the same point (KATZ 1939).

This discontinuity need not be considered in the present study since the plateau of full activity is developed within a few msec at the end of the mechanical latent period (HILL 1951 a, ABBOTT and RITCHIE 1951 b) and since the load on the fibres did not exceed P_o during the vibrations (cf. p. 201). Thus, in terms of HILL's three-component model the contractile component shortened during the entire oscillating course of shortening of the fibre bundle and no phsyiological lengthening of the contractile component occurred.

The shortening velocity of the fibre bundle was zero whenever the series elastic component during its oscillations lengthened at the same speed as that with which the contractile component shortened.

(4) HILL (1953 a) states: Except for very small extensions an active muscle rapidly stretched "gives", and the work done on it is largely degraded into heat; to describe the effect of this as "damping" obscures the issue.

With the small vibrational amplitudes there were, however, no signs of a "give" at the loads employed in the present experiments. Besides, a "give" would reduce the stiffness and therefore partly conceal the increase in stiffness caused by the stimulus.

(5) HILL (1953 a) finally states: "under all conditions the presence of the series elastic element adds a complication difficult, or impossible, to disentangle; indeed with a high frequency and a small amplitude of alternation it would probably provide the chief effect". It is implied in this argument that vibration experiments can give only limited information about the contractile substance. This conclusion seems, however, hardly justified.

In HILL's three-component muscle model the contractile component is characterized solely by the characteristic relation between shortening velocity and load, the elasticity of the contractile substance being disregarded. This formal definition of the contractile component implies that the elasticity of the contractile elements is attributed to the series elastic component of the muscle model.

That this component is not solely localized in the tendinous tissue is indicated by direct microscopical measurements on the frog's sartorius during isometric contraction. JEWELL and WILKIE (1958) found in the tibial end of the tetanically contracted muscle the stretch of the tendon to be negligible and in the pelvic end a stretch of the tendon of 0.1—0.25 mm. Since release experiments indicated that the series elastic component was stretched by at least 0.5 mm, more than half of the series elasticity must be localized in the muscular tissue itself. The length changes observed by JEWELL and WILKIE (1958) in different parts of the muscle tissue during the development of the isometric tetanus indicate that the part of the series elastic component which resides in the muscle tissue is homogeneously distributed along the major part of the muscle length.

When the periodical length changes in vibration experiments according to Hill's three component model mainly occur in the series elastic component it may seem contradictory that in spite of the increase in dynamic stiffness during isotonic contraction no change has been found in the stress-strain relationship of the series elastic component during the rising phase of an isometric twitch (WILKIE 1956 a, b, JEWELL and WILKIE 1958). However, the stress-strain relation was measured 100 msec or more after the stimulus (0° C), *i. e.* at a time when the changes in dynamic stiffness are much less pronounced than in the initial phase of the contraction.

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Vitamin D and Calcium Absorption in Parathyroidectomized Rats

By

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Abstract

GRAN, F. C. *Vitamin D and calcium absorption in parathyroidectomized rats.* Acta physiol. scand. 1960. *49.* 211—215.—The absorption of calcium has been studied in parathyroidectomized rats. The destruction of the parathyroid glands did not affect the absorption of Ca. The absence of the parathyroid hormone did not impair the response of the Ca absorption to the administration of vitamin D.

A striking feature in the physiology of Ca absorption is the adaptation of the efficiency according to the requirements of the body, provided that vitamin D is present. The adaptation depends upon an undersaturation of the skeleton with Ca, and this led NICOLAYSEN (1943) to suggest that an endogenous factor of unknown origin is released, which in turn promotes the absorption of Ca from the intestine (NICOLAYSEN, EEG-LARSEN, and MALM 1953, NICOLAYSEN and EEG-LARSEN 1953, and HAAVALDSEN, MORTENSEN EGNUND, and NICOLAYSEN 1956).

It is well established that the parathyroid hormone has a direct action on bone (MCLEAN, chapter 22 in BOURNE 1956, NEUMAN and NEUMAN 1958, MCLEAN and BUDY 1959); an excess of the parathyroid hormone promotes demineralization of the skeleton. In humans with parathyroid adenomas there is a high efficiency of Ca absorption (BAUER, ALBRIGHT, and AUB 1930, AUB, TIBBETTS, and MCLEAN 1937, and ROBERTSON 1942). On the other hand, injections of parathyroid extracts did not increase the Ca absorption (STEWART and PERCIVAL 1927, ALBRIGHT *et al.* 1929, BÜLBRING 1931, ALBRIGHT and

REIFENSTEIN 1948). Parathyroidectomy did not influence the Ca absorption (ALBRIGHT and REIFENSTEIN 1948), in one case a young boy showed a remarkable ability to retain Ca (BAUER *et al.* 1930).

The following investigation was undertaken in order to elucidate if the parathyroid glands possibly could be related to the endogenous factor of NICOLAYSEN (1943). More information was also needed with regard to the specific mode of actions of vitamin D and the parathyroid glands.

Methods

Hooded and albino rats of both sexes were taken from the strains of the Institute. Parathyroidectomy was carried out by careful cauterization of the glands, the operational technique necessarily resulted in a partial thyroidectomy. The rats were anesthesized with ether. The success of the operation was checked by repeated determinations of serum Ca (GRAN 1960 a), blood samples for the analyses were taken from the tail.

The composition of the diets have been reported elsewhere (GRAN 1960 b), the pre-experimental diet and the diet used for the determinations of digestive juices Ca were used. The diets and water were given to the animals *ad libitum.* The rats received in addition a weekly supplement of 140 I. U. vitamin A acetate, a weekly addition of 70 I. U. vitamin D was given when desired.

The rats were deprived of vitamin D over a considerable length of time in advance of the experiments in order to ensure a complete lack of vitamin D. The pre-experimental diet (GRAN 1960 b) with 0.25 per cent Ca and 0.4 per cent P was given during this period.

The experimental diets were given for the first time two days in advance of the actual experiments. The rats were maintained in individual cages which allowed separate collections of faeces without contamination by food or urine. The length o the metabolic periods in each experiment is indicated in the tables.

The Ca determinations were carried out by the precipitation of Ca with oxalate followed by titration with standard potassium permanganate, on samples of food and faeces digested with nitric and perchloric acids. The dietary intake was determined in the metabolic periods by the use of chromic oxide as a marker (GRAN 1960 b).

Experimental

The Ca absorption was studied in parathyroidectomized rats in several experiments, of which two will be reported here. The general experimental plan was to deplete the animals of their body stores of vitamin D during a pre-experimental period which was extended over at least two months, in which the animals were given a diet with 0.25 per cent Ca and 0.4 per cent P. When the rats were taken into the experiments, the Ca absorption was studied over several consecutive metabolic periods without vitamin D, next the rats were given a weekly dose of 70 I. U. vitamin D in order to study the response of the Ca absorption to this administration.

In the first experiment the rats were parathyroidectomized two months ahead of the experiment, vitamin D was not given during this time. Six male

Table I. Calcium absorption in parathyroidectomized rats, and the response to the administration of vitamin D

Six male rats, 6 months old, were parathyroidectomized two months in advance of the experiment. The pre-experimental diet contained 0.25 per cent Ca and no vitamin D. This diet was given from the time of operation until the start of the experiment. The experimental diets contained 0.4 per cent P.

Period no.	Duration days	Per cent Ca in the diet	Vitamin D given	Body weight g	Serum-Ca mg/100 ml	Ca-intake mg/day	Ca-absorption mg/day
1	14	[1]0.041	No	197	5.9 ± 0.3	3.3 ± 0.3	− 0.5 ± 0.9
2	14	[1]0.041	No	200		3.4 ± 0.1	0.2 ± 0.5
3	14	[1]0.041	No	199	6.6 ± 0.3	4.2 ± 0.4	0.4 ± 0.9
4	14	[1]0.041	No	194		4.1 ± 0.3	0.2 ± 0.8
5	7	[1]0.041	No	194	4.1 ± 0.2	3.9 ± 0.2	0.4 ± 1.0
6	7	[1]0.041	[2]Yes	186		4.4 ± 0.5	1.4 ± 1.2
7	7	[1]0.041	Yes	201	6.0 ± 0.3	5.1 ± 0.1	2.7 ± 1.3
8	7	0.235	Yes	194		14.8 ± 0.8	6.8 ± 4.1
9	7	0.235	Yes	184	6.2 ± 0.4	25.6 ± 1.5	15.0 ± 3.9
10	7	0.235	Yes	208		26.9 ± 2.9	10.9 ± 4.1
11	7	0.235	Yes	222	6.3 ± 0.3	23.1 ± 0.8	10.4 ± 1.8

[1] 0.5 per cent ammonium oxalate was added to this diet.
[2] 70 I.U. vitamin D_2 weekly.

The figures are mean ± standard error of the mean.

rats were used, they were 6 months old when the actual experiment started. A diet with 0.041 per cent ammonium oxalate was first given for 9 weeks in order to deplete the rats for some of their body Ca. Next the rats received vitamin D. The low Ca diet was given for two additional weeks followed by a diet with 0.235 per cent Ca and 0.4 per cent P.

In the second experiment, ten 17-month-old female rats were used. These animals had not been allowed vitamin D for the six months prior to the experiment. In this experiment the Ca absorption was first studied for two metabolic periods whereafter the parathyroid glands were destroyed. The Ca absorption was studied for additional 20 days without vitamin D, and for 28 days after the vitamin D had been administered. The experimental diet contained 0.235 per cent Ca and 0.4 per cent P.

Results

The results are found in Tables I and II. A striking increase in the absorption of Ca followed immediately after the adminstration of vitamin D in Table II. The results in Table I indicate the same response; when Ca in the diet was increased and oxalate was removed, the Ca absorption immediately became

Table II. Calcium absorption in rats related to the activity of the parathyroid glands and vitamin D

The rats were 17-month-old females. The animals had been given a diet with 0.25 per cent Ca and 0.4 per cent P without vitamin D for 6 months before they were taken into the experiment. The time of parathyroidectomy and start of vitamin D administration is indicated in the table. The experimental diet contained 0.235 per cent Ca and 0.4 per cent P.

Period no.		Duration days	Number of rats	Body weight g	Serum Ca mg/100 ml	Ca-intake mg/day	Ca-absorption mg/day
1	No vitamin D	4	10	268		19.6 ± 0.8	-0.9 ± 0.4
2		4	10	264	7.5 ± 1.0	29.5 ± 0.9	0.8 ± 0.7
3	Parathyroid-	4	10	259		6.8 ± 1.9	-1.8 ± 1.3
4	ectomized, no	8	10	257		16.5 ± 1.6	0.1 ± 0.9
5	vitamin D	8	9	221	3.6 ± 0.3	26.7 ± 1.8	0.5 ± 0.5
6	Plus 70 I.U.	4	7	218	3.9 ± 0.7	28.9 ± 1.6	6.8 ± 0.5
7	vitamin D_2	4	7	231		34.4 ± 3.4	12.7 ± 2.7
8	per week	4	7	240		31.2 ± 1.1	9.1 ± 0.7
9		4	7	242	5.0 ± 0.4	33.0 ± 1.2	9.4 ± 0.8
10		4	7	249		34.3 ± 0.8	6.5 ± 0.9
11		4	7	247		22.1 ± 1.9	3.7 ± 0.6
12		4	7	247	5.1 ± 0.8	34.9 ± 1.5	7.4 ± 0.8

The figures are mean $\pm$ standard error of the mean.

high. The Ca absorption was low in the absence of vitamin D and the removal of the parathyroids apparently did not affect the Ca absorption. It appears from Table II that the very low serum Ca in the parathyroidectomized rats did not result in any significant increase in the Ca absorption. This strengthens the view of NICOLAYSEN *et al.* (1953) that the level of serum Ca is not the important factor.

The parathyroidectomy resulted as expected, in a fall of the serum Ca. The administration of vitamin D resulted in a slight increase of serum Ca but the level remained very low.

Discussion

The results are in agreement with HAAVALDSEN *et al.* (1956) with regard to the action of vitamin D on the Ca absorption. The parathyroid glands appear to be unnecessary for the regulation of Ca absorption from the intestine; in consequence the endogenous factor of NICOLAYSEN (1943) cannot be identical to the parathyroid hormone. Unpublished experiments carried out by LÖNNUM in this laboratory indicate that the adaptation of the Ca absorption in the rat also is independent of the adrenals and the testicles.

Earlier experiments in rats with intact parathyroids in this laboratory (Haavaldsen *et al.* 1956) indicated heavy Ca losses in the faeces following oxalate ingestion. The very low digestive juices Ca in the parathyroidectomized rats as compared with others (Gran 1960 b), may explain the difference in the results achieved in rats with and without parathyroids.

The high rate of Ca absorption observed in patients with parathyroid adenomas, most likely is a result of the skeletal undersaturation of Ca caused by the bone resorption brought about by the excess of parathyroid hormone. A sufficient degree of skeletal demineralization was probably not obtained following injections of parathyroid extracts (for references, see introduction), and this may explain why such injections have failed to increase the Ca absorption.

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Acta physiol. scand. 1960. 49. 216—223

From the Research Laboratories of the State Alcohol Monopoly (Alko), Helsinki, Finland

Comparison of the Effect of Ethanol and Malonate on the Respiration of Rat Brain Cortex Slices

By

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Abstract

WALLGREN, H. *Comparison of the effect of ethanol and malonate on the respiration of rat brain cortex slices.* Acta physiol. scand. 1960. *49*. 216—223. — Ethanol and malonate both reduce the respiration of stimulated cerebral cortex tissue at concentrations which do not depress the oxygen uptake of unstimulated tissue. A study was made of the effects of these compounds added separately or simultaneously at low concentrations to a phosphate-glucose saline. Within the concentration ranges employed (malonate $10^{-4} - 8 \times 10^{-4}$M, ethanol $8.7 \times 10^{-2} - 1.96 \times 10^{-1}$M), the only effect on unstimulated tissue was a slight increase in respiration in presence of ethanol. In electrically stimulated tissue, malonate and ethanol both diminished the respiration. When the inhibitors were present simultaneously, the effect of one of them only was obtained, *i. e.* of that inhibitor having greater action at the particular concentration. This lack of synergism was taken as evidence for independent inhibitory action of ethanol and malonate.

When isolated cerebral cortex tissue is stimulated with potassium ions or electrical pulses, it respires at a high rate which requires the presence of glucose, pyruvate or lactate as substrate (McILWAIN 1953), and which is depressed by low concentrations of malonate (KIMURA and NIWA 1953, TSUKADA and TAKAGAKI 1955, HEALD 1953, QUASTEL 1959) and also by ethanol at pharmacologically active concentrations (GHOSH and QUASTEL 1954, FISCHER 1957, BEER and QUASTEL 1958, WALLGREN and KULONEN 1960). In view of these observations, it has been suggested that ethanol may act on a specific phase of nerve respiration which is involved in the response

Table I. Combinations of experimental conditions employed

The symbols signify: C controls; E medium containing ethanol; M medium containing malonate; ME medium containing both malonate and ethanol. Each symbol corresponds to a pair of vessels, one of which was used for observation of the respiration of unstimulated tissue and the other for that of stimulated tissue. The pairs of vessels matched contained tissue slices obtained from the same brain. The numerals indicate the number of experiments successfully conducted in the conditions indicated.

C	M	C	E	M	ME	E	ME
18	18	19	19	15	15	15	16

to stimulation and which is concerned with glucose or pyruvate metabolism, and perhaps particularly with the citric acid cycle (Ghosh and Quastel 1954, Quastel 1957). Since then, it has been demonstrated that the respiration of brain mitochondria is not sensitive to alcohol at "physiological" concentrations (Beer and Quastel 1958), a finding which renders the above hypothesis less plausible.

In an earlier study from these laboratories (Wallgren and Kulonen 1960) the effect of ethanol on the respiration of brain cortex slices in the presence of varying concentrations of glucose was reported. Since the action of ethanol was independent of glucose concentration, it was considered probable that ethanol does not inhibit glycolysis competitively. The same expedient, *i. e.* varying substrate concentration, could not be employed for the investigation of possible ethanol competition at some stage of the citric acid cycle, since none of the intermediates in the cycle can by itself support the respiratory response to electrical pulses (McIlwain 1953). Another approach would be to use some inhibitor. The present investigation aimed at obtaining further information on the relationship between ethanol-induced depression of respiration in nerve tissue and the action of malonate. It was decided to compare the effects of separate and simultaneous addition of malonate and ethanol to the experimental medium.

Experimental

All experiments were performed with conventional Warburg constant-volume respiratory manometers. A detailed description of the apparatus for electrical stimulation of tissue slices and of the preparation of the slices has been given previously (Wallgren and Kulonen 1960).

Phosphate-glucose saline (McIlwain 1951) with 6 mM glucose was used throughout, the volume per vessel being 2.5 ml. The flasks were gassed with pure oxygen and the experimental temperature was 37.5° C except when otherwise stated.

Adult rats of the laboratory stock were used. The animals were sacrificed by decapitation in a cold room where the brain cortex slices were prepared. After transfer of the slices to the chilled vessels, these were placed in the thermostat and shaking (96

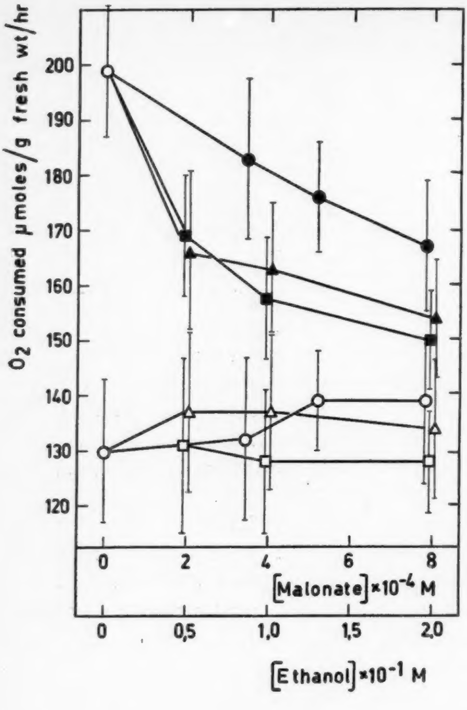

Fig. 1. Effect of malonate and ethanol on respiration of rat brain cortex slices incubated in phosphate saline at 37.5° C with 6 mM glucose. Open symbols, unstimulated; black symbols, stimulated. (□), with malonate; (○), with ethanol: (△), with 1.3×10^{-1}M ethanol and 2×10^{-4}M malonate, and 1.96×10^{-1}M ethanol and 4 or 8×10^{-4}M malonate. Vertical bars indicate standard deviations.

complete strokes/min) commenced. After gassing had proceeded for 3 min, the required solutions of inhibitors, dissolved in 0.2 ml of experimental saline, were placed in the side arms of the vessels. After 10 min equilibration, the inhibitor solutions were tipped into the main compartment of the flasks and stimulation was initiated by passing condenser pulses between concentric electrodes at a frequency of 180—200/sec and a voltage gradient of 1.6—1.8 V/mm. After an additional 5 min (18 min after transfer to the bath) the first manometer reading was taken and observations were made for one hour at 10 min intervals.

All experiments were set up with 2 standard flasks and 2 electrode vessels. From each brain hemisphere, one slice (25—40 mg) was used for determination of the respiration of unstimulated tissue and two slices (50—70 mg) for that of stimulated tissue. The two pairs of vessels used in each experiment were thus provided with slices obtained from different lobes of the same brain and subject to different conditions with respect to the inhibitor present. Table I shows how the addition of inhibitors was distributed between the pairs of flasks. The following concentrations of the inhibitors were used: malonate (throughout as the sodium salt) 2×10^{-4}M (13 experiments), 4×10^{-4}M (10 expts.), 8×10^{-4}M (10 expts.); ethanol 1.3×10^{-1}M (13 expts.), 1.96×10^{-1}M (21 expts.); malonate 2×10^{-4}M with ethanol 1.3×10^{-1}M (10 expts.), malonate 4×10^{-4}M with ethanol 1.96×10^{-1}M (10 expts.), malonate 8×10^{-4}M with ethanol 1.96×10^{-1}M (10 expts.). The values with 8.7×10^{-2}M ethanol (Fig.

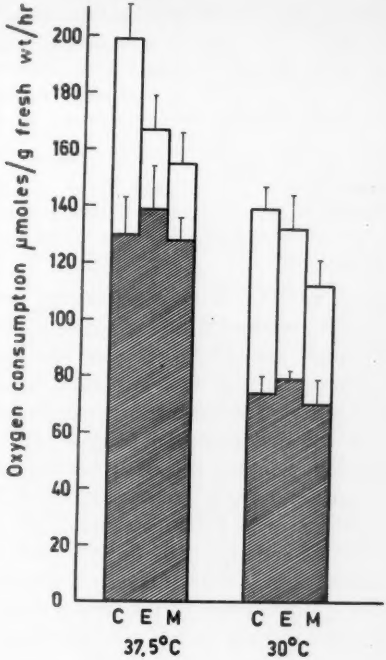

Fig. 2. Oxygen uptake of brain cortex tissue from rat during one-hour experiments at 37.5° and at 30° C. C = control, E = 1.96×10^{-1}M ethanol, M = 4×10^{-4}M malonate. Shaded area = unstimulated tissue, white area = increase in respiration induced by electrical pulses. Positive standard deviations indicated.

1) are taken from our earlier report (WALLGREN and KULONEN 1960). It was considered justified to include them since the control series in the present and the earlier experiments did not differ significantly. Supplementary experiments not included in Table I were performed in the following conditions: 10^{-4}M malonate (12 expts.); 10^{-4}M malonate and 8.7×10^{-2}M ethanol (13 expts.); 10^{-4}M malonate and 1.96×10^{-1}M ethanol (10 expts.). Since controls were not used in all experiments with inhibitors, comparisons were based on the absolute values obtained and analysis of the significance of the difference between means was performed by means of Student's t-test.

At 30° C, 10 experiments with 4×10^{-4}M malonate and 10 experiments with 1.96×10^{-1}M ethanol were performed, using one hemisphere from each brain as a control and the other hemisphere for observations on the effects of the inhibitors. The t-test for non-independent means was used in statistical evaluation of the results of these experiments.

Results

The relationship of oxygen consumption to inhibitor concentration is shown in Fig. 1. In unstimulated tissue, the only significant effect was the increase in respiration in the presence of ethanol (with 1.3×10^{-1}M, $P < 0.05$, and with 1.96×10^{-1}M, $P < 0.025$). In stimulated tissue, all inhibitor concentrations caused significant ($P < 0.001$) reduction of respiration. The depressing

Table II. Q_{10} of oxygen uptake of brain cortex slices

The slices were incubated in control medium, in the presence of malonate (4×10^{-4}M) and in the presence of ethanol (1.96×10^{-1}M). Experiments were performed at 30° and 37.5° C and the quotients have been calculated from the data in Fig. 2.

	Control	Malonate	Ethanol
Unstimulated	2.01	2.11	2.03
Stimulated	1.57	1.52	1.36

action of ethanol appeared to be proportional to its concentration in the medium. Malonate already had a surprisingly large effect at 2×10^{-4}M. Although the inhibition was significantly greater ($P < 0.001$) when the concentration was increased to 8×10^{-4}M, it was possibly sufficiently large at 2×10^{-4}M to obscure any additive effects of ethanol present simultaneously with the malonate. To control this point, the supplementary experiments mentioned in the preceding section were performed. The results, expressed as μmoles oxygen consumed/g fresh tissue/hr. $\pm$ standard deviation, were as follows: with 10^{-4}M malonate 3.88 ± 0.37; with 10^{-4}M malonate and 8.7 × × 10^{-4}M ethanol 3.84 ± 0.27; with 10^{-4}M malonate and 1.96×10^{-1}M ethanol 3.70 ± 0.33. Oxygen consumption with 1.96×10^{-1}M ethanol was 3.75 ± 0.27. Thus, in the presence of both ethanol and malonate, the effect obtained did not deviate significantly from that observed with either malonate or ethanol alone, depending on which of them had a larger inhibiting action at the particular concentration.

In Fig. 2 is shown the average oxygen consumption at 37.5° C and at 30° C of control tissue and of tissue incubated in the presence of either 4×10^{-4}M malonate or 1.96×10^{-1}M ethanol. The effect of stimulation was proportionally greater at the lower temperature. At both temperatures, ethanol increased the respiration of unstimulated tissue (30°, $P < 0.05$). The effect of malonate was insignificant. Both inhibitors had a marked effect on stimulated tissue at 37.5°, whereas at 30° the ethanol effect was much reduced, although still significant ($P < 0.05$). Since inhibition was obtained at 30°, the Q_{10} of the oxygen consumption in control conditions could be compared with that in the presence of malonate or ethanol (Table II). In unstimulated tissue, the Q_{10}s were virtually identical, whereas ethanol clearly depressed the Q_{10} of stimulated tissue.

Rate of oxygen consumption was plotted against time (Fig. 3). In control conditions, oxygen consumption fell off more rapidly at 37.5° C than at 30° C. When inhibitor (4×10^{-4}M malonate or 1.96×10^{-1}M ethanol) was present, no such differences between the two temperatures were observed. The full effect of the inhibitor was obtained during the first period of manometer reading, and the action appeared to remain unchanged throughout the experimental period.

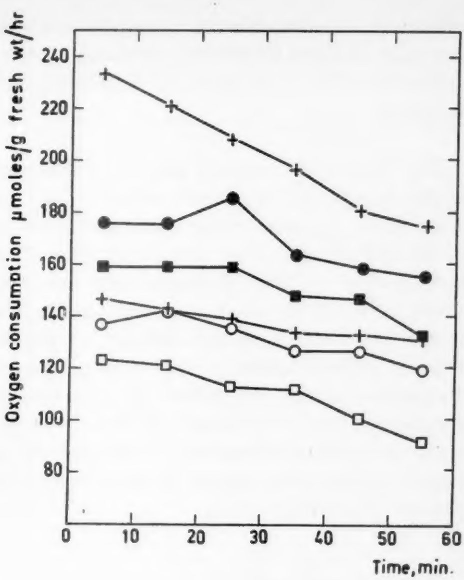

Fig. 3. Rate of oxygen consumption of electrically stimulated rat brain cortex tissue respiring in phosphate-glucose saline at 37.5° (black symbols) and 30° C (open symbols). (+), control (upper 37.5°); (○) in presence of 1.96×10^{-1}M ethanol; (□) in presence of 4×10^{-4} M malonate.

Discussion

The results reported here conform with earlier reports (Kimura and Niwa 1953, Tsukada and Takagaki 1955, Heald 1953) on the high sensitivity to malonate of stimulated cerebral cortex slices. The present preparations were even more sensitive than those used by other workers. At the low concentrations of malonate employed in the present experiments, only succinic dehydrogenase should be inhibited (Pardee and Potter 1949). Whether the lack of effect of low concentrations of malonate in unstimulated tissue should be taken as evidence of qualitative differences in the metabolism of stimulated and unstimulated tissue, or as a consequence of a lower and therefore less vulnerable respiratory rate supported by the same metabolizing system (Brink 1957) is still a matter for conjecture.

In the concentration range employed in the present experiments, ethanol and malonate have effects which differ in several respects. Ethanol slightly augments the respiration of unstimulated tissue, whereas malonate does not. The depression in the presence of ethanol in stimulated tissue is greatly reduced by fall in temperature, that in the presence of malonate only slightly. There is a great difference in the concentrations of the two agents required to produce the same fall in oxygen uptake. Actually, in order to obtain an

effect with ethanol, one has to employ concentrations much in excess of those generally required for specific inhibition of enzymatic reactions. These features suggest a different target and a different mode of action for ethanol than for malonate.

The absence of synergism between the two compounds lends further support to this hypothesis. Apparently ethanol does not exert its action on stimulated brain slices by interference with any part of the enzyme system involved in the dehydrogenation of succinic acid. The result is in line with Beer's and Quastel's (1958) demonstration that respiration of brain mitochondria is not inhibited by alcohol at pharmacologically active concentrations. The present observations do not exclude the possibility of interference by ethanol in some other reaction in the citric acid cycle, although, in the opinion of the present author, the differences in action of malonate and ethanol render it unlikely. Direct inhibition of some other oxidative pathway does not seem a very plausible explanation of the observed effects of ethanol, since the response to stimulation appears to be intimately linked with oxidation of pyruvate through the Krebs' cycle.

The author is indebted to Mrs. Kaija Salmela and Mr. Ralph Lindbohm for their assistance in the experiments.

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Excretion of Intravenously Injected Solid Particles in Bile

By

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Abstract

JUHLIN, L. *Excretion of intravenously injected solid particles in bile.* Acta physiol. scand. 1960. *49.* 224—230. — Spherical hydrophilic particles of methyl methacrylate marked with a fluorescent colour were injected i. v. in rabbits, and their concentration in blood and bile was estimated during the following hours. Three sizes of particles were used. After injection of 192 mg particles ranging in diameter from 0.02 to 0.11 μ, particles appeared in the bile in a concentration of 0.05—0.40 mg/ml during the first minutes after i. v. injection. A relative blockade of the R. E. S. by pretreatment with nonfluorescent particles decreased the rate of clearance of the particles from the blood. This increase in particles in blood was not accompanied by a corresponding increase in the bile; instead there was a tendency to a decrease in biliary particle excretion. There was almost no biliary excretion of 0.06—0.14 μ particles. The lowest detectable concentration of these particles is 0.004 mg/ml bile. Particles 0.2—0.8 μ in diameter (detectable in amounts over 0.04 mg/ml) could not be found in the bile even with doses up to 1,200 mg. The route of passage of particles to the bile is discussed.

By electronmicroscopic observation HAMPTON (1958) showed that intravenously injected particles of Thorotrast® and HgS were discharged into the bile capillaries. He demonstrated that the particles entered the perisinusoidal space of DISSE through large discontinuities in the sinusoidal epithelium where they were taken up by the hepatic cells. The particles were then transported through these cells to the bile capillaries in vacuoles formed by invagination of the surface. HAMPTON failed to confirm ROUILLER's (1956)

observations that there should be a direct communication between the perisinusoidal space of DISSE and the bile capillaries. For a review of earlier literature on this subject see HAMPTON (1958).

In this investigation we used spherical fluorescent plastic particles prepared in different batches, with rather homogeneous size ranging from 0.02 μ to 1.2 μ, to study the influence of the size of the particles on their excretion through the bile after intravenous injection. Our aim was further to study the relation between particle concentration in blood and excretion through bile as well as the influence of so called "reticulo-endothelial blockade" by previous injection of similar nonfluorescent particles.

Methods

Animals

Male albino rabbits weighing 2.0—2.3 kg were used. Before the experiments they were fed on hay, oat, water ad lib.

Particles

The particles were prepared by emulsion polymerisation of methyl methacrylate and marked with a fluorescent dye as described earlier (JUHLIN 1956, 1959). The following sizes of hydrophilic and negatively charged particles were used:

Size I Mean diameter 0.07 μ. Range 0.02—0.11 μ. (Prep. 2, JUHLIN 1959)
Size II Mean diameter 0.10 μ. Range 0.06—0.14 μ. (Prep. Sh, JUHLIN 1956)
Size III Mean diameter 0.8 μ. Range 0.2—0.8 μ. (Prep. Lh, JUHLIN 1956)

Experimental procedure

The rabbits were anaesthetized by intravenous injection of 0.6 ml/kg Numal® (Roche). A fine plastic tube 10 cm in length was inserted into the hepatic duct and fastened with a silk ligature. The cystic duct was ligated. The bile volume in the tube was about 0.01 ml. It was ascertained that the bile was clear and absolutely free from blood. Another larger plastic tube was inserted into the femoral artery and fixed in position to permit collection of blood samples for regular estimation of particles in the blood.

After 2 ml of bile had been collected, the particle suspension was injected intravenously into the marginal vein of the pinna. The injections were either completed within 2 min or given as continuous injections with an apparatus described by ÖBRINK (1948). The suspension injected contained 30 mg/ml of solid particles in 5 % sterile glucose solution.

Estimation of particles in blood and bile

One millilitre of blood or bile was added to 10 ml of ethylacetate and shaken several times during 1 hour. This procedure dissolved the particles and the fluorescent colour. The fluorescence was then estimated in a Coleman Electronic Photofluorometer (JUHLIN 1958). Two blank specimens of the bile were taken in each experiment before the injection of fluorescent particles and were always found to be similar. The subsequent estimations were corrected for this blank value. In experiments where particles without fluorescent colour were injected, the bile's own fluorescence was constant through the whole experimental period (1—4 hours). The least detectable amounts were 4 μg/ml of sizes I and II and 40 μg of size III.

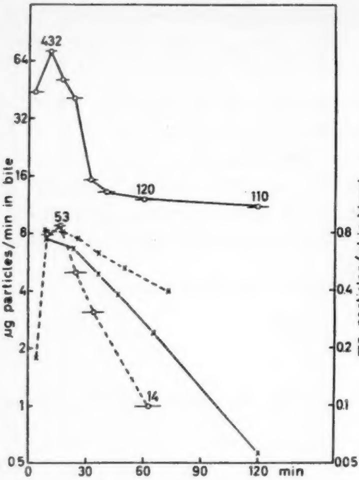


Fig. 1

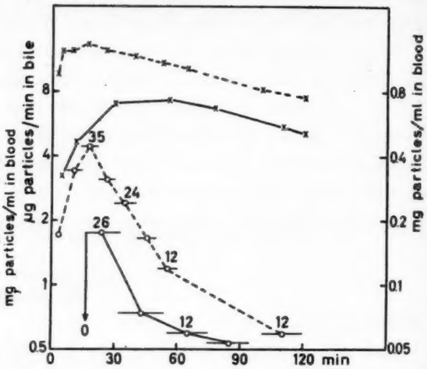


Fig. 2

Fig. 1. Concentration of particles in blood and bile after intravenous injection of 192 mg 0.02—0.11 μ particles. (Highest and lowest bile curve in 4 experiments.)
Abscissa: Time in minutes after injection of particles.
Left ordinate: Particles μg/min in bile. The horisontal line indicates the time during which the bile (1 ml) was collected and the numbers the concentration in μg/ml bile.
Right ordinate: Particles, mg/ml blood.

Exp. 1. Blood ×———×———× Exp. 2. Blood ×— — —×— — —×
Bile O———O———O Bile O— — —O— — —O

Fig. 2. Concentration of particles in blood and bile of rabbits pretreated with 192 mg non-fluorescent particles 30 min before injection of 192 mg fluorescent 0.02—0.11 μ particles. (Highest and lowest bile curve in 4 experiments.)
Abscissa and *ordinate* as in Fig. 1.

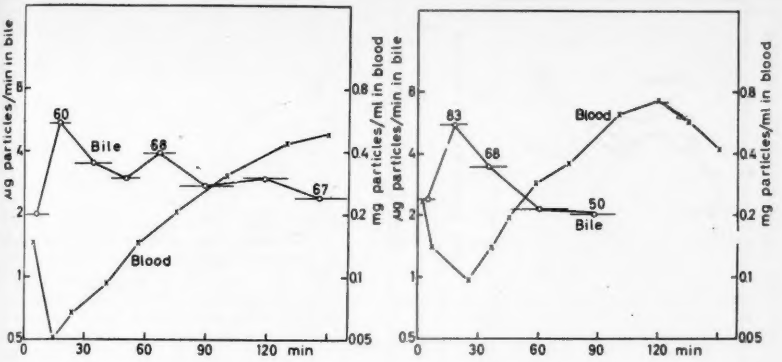
Exp. 5. Blood ×———×———× Exp. 6. Blood ×— — —×— — —×
Bile O———O———O Bile O— — —O— — —O

Results

1. Size I particles (0.02—0.11 μ) injected i. v. into rabbits in a dose of 96 mg disappeared from the blood stream within 30 min and appeared in bile in barely measurable amounts 10—30 min after injection. With a particle dose of 192 mg, particles appeared in the bile during the first minutes after injection (Fig. 1).

Pretreatment 30 min earlier with 192 mg of similar particles without fluorescence decreased the blood disappearance rate of a subsequent fluorescent particle injection of 192 mg. The increase of particles in the blood was not followed by a corresponding increase in the bile. Instead there was a tendency to a decrease in biliary particle excretion (Fig. 2).

Following pretreatment with larger doses (384 mg) of uncoloured particles 60 and 30 min before injection of fluorescent particles (192 mg) the blood



a) b)

Fig. 3. Concentration of 0.02—0.11 μ particles in blood and bile.
a) Rabbit pretreated with 192 mg nonfluorescent particles 60 and 30 min before injection of 192 mg fluorescent particles.
b) Rabbit administered 384 mg particles by injection during 5 min. Bile flow stopped after 100 min.
Abscissa and *ordinate* as in Fig. 1.

concentration of fluorescent particles, after an initial decrease, showed an increase after 15 min (Fig. 3 a). This was found to be due to a flocculation of the particles in the lung. The amount of particles excreted per minute in the bile did not follow the blood particle concentration curve (Fig. 3 a). Similar results were obtained when particles in a dose of 384 mg were injected during 5 min without pretreatment (Fig. 3 b).

When 192 mg of the fluorescent particles was injected 3 times at 30 min intervals, the blood concentration was held at a more constant level. The excretion through the bile was of about the same magnitude as in non-pretreated animals (Fig. 4), but the individual experiments varied rather widely. This is evident from Fig. 5, where the biliary excretion of particles after repeated injections was followed during several hours without estimation of the particles in the blood.

2. After injection of size II particles (0.06—0.14 μ) in a dose of 192 mg no particles or very small amounts were excreted through the bile (Fig. 6). With repeated doses of 192 mg or pretreatment with non-fluorescent particles, fairly high concentrations of particles could be obtained in the blood, but the biliary excretion was less than with the particles of 0.02—0.11 μ (Fig. 6 and 7).

3. When particles 0.8 μ in diameter were injected i. v., they disappeared rapidly from the blood stream and no particles could be found in the bile even with a single injection of 600 mg or repeated doses up to a total of 900 mg during 90 min. Continuous injection of 1,200 mg during 1 hour gave a concentration of particles in the blood of about the same magnitude as that ob-

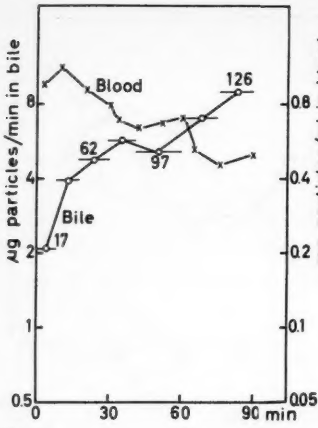


Fig. 4

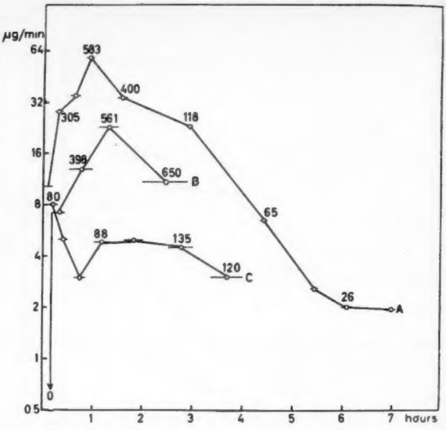


Fig. 5

Fig. 4. Concentration of particles in blood and bile after injection of 192 mg 0.02—0.11 μ particles at 0.30 and 60 min.
Abscissa: Time in minutes after first (0 time) particle injection.
Ordinate: see Fig. 1.

Fig. 5. Concentration of particles in bile after injection of 192 mg 0.02—0.11 μ particles at 0 and 30 min (curve A) and at 0, 30, 60 and 90 min (curves B and C).
Abscissa: Time in hours after first particle injection.
Ordinate: Particles μg/min in bile. The horisontal line indicates the time during which the bile (1 ml) was collected.

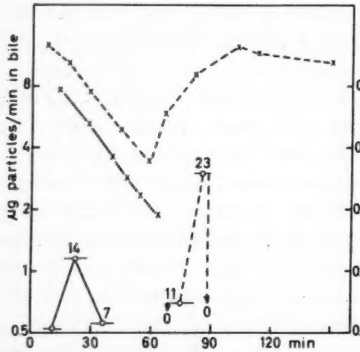


Fig. 6

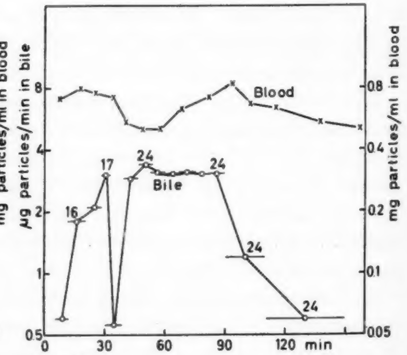


Fig. 7

Fig. 6. Concentration of particles in blood and bile after injection of 192 mg 0.06—0.14 μ particles. In experiment 2 no particles were found in the bile during 0—60 min, wherefore 192 mg was again injected at 60 min.
Abscissa and *ordinate* as in Fig. 1.

Exp. 1. Blood ×———×———×
Bile O———O———O

Exp. 2. Blood × — — — × — — — ×
Bile O — — — O — — — O

Fig. 7. Concentration of 0.06—0.14 μ particles in blood and bile of rabbits pretreated with 192 mg of nonfluorescent particles 30 min before injection of 192 mg particles at 0 and 30 min and 96 mg at 90 min.

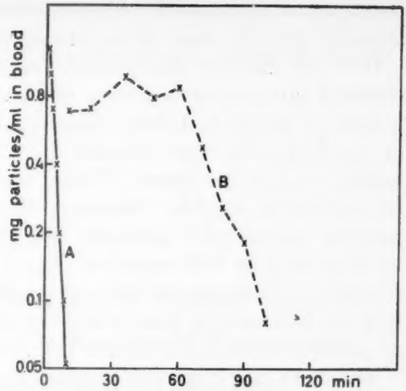


Fig. 8. Concentration of 0.2—0.8 μ particles in blood after a single injection of 600 mg (A) and continuous injection of 1,200 mg during 0—60 min (B). No particles could be demonstrated in bile.
Abscissa: Time in minutes after end of single injection or start of continuous injection.
Ordinate: Particles, mg/ml blood.

tained with the smaller particles. Even then, no particle fluorescence could be found in the bile (Fig. 8).

Discussion

Size I particles, ranging from 0.02 to 0.11 μ, were more easily transported to the bile than size II particles (0.06—0.11 μ). Size III particles with a size range from 0.2 to 1.3 μ did not appear in the bile. The maximum size of particles found to pass in detectable amounts from the liver sinusoids to the bile seems therefore to be about 0.1 μ. The sensitivity of the method used to detect the particles in bile is limited, however, and, if less than 40 μg/ml of the large particles was excreted per minute in bile, they would not have been detected.

Hampton (1958) showed that 2 days after i. v. injection of Thorotrast® there were large aggregates of particles about 1 μ in diameter in the hepatic cells. Hampton thought that the particles were temporarily stored here until they could be excreted via the bile channels. Why then do smaller particles (0.02—0.11 μ) pass more easily to the bile? One possibility is that the 0.2—0.8 μ particles are too large to be able to pass through the discontinuities of the sinusoidal wall into the perisinusoidal space of Disse or if some of the particles have reached the space of Disse, they are immediately phagocytized there by the RE-cells. Another possibility is, of course, that the larger particles are taken up or delivered with greater difficulties by the hepatic cells.

If the space of Disse communicates directly with the bile canaliculi, as supposed by Rouiller (1956), the explanation could be that these communications are small enough to impede the larger particles. If such preformed channels exist, on the other hand, one would have expected that a higher con-

centration of particles in the blood would be reflected in a corresponding increase in the bile. Such is not always the case, however.

It is well known, that elimination from the blood stream is delayed by repeated intravenous injections of particles, due to a decreased phagocytic activity of the R. E. S. We, therefore, thought that if the phagocytic activity of the R. E. cells were blocked more particles would be available for the hepatic cells in the space of Disse, which should give an increased amount of particles in the bile. However, when the pretreatment with uncoloured particles consists of a moderate dose (192 mg), there is merely a tendency to a decrease in bile excretion (Fig. 1 versus Fig. 2) and the highest concentration of particles in bile is always found in non-pretreated animals. Thus it seems that particle pretreatment results in a partial blockade of the uptake or passage through the hepatic cells.

When the larger doses of particles are injected during a short time into the marginal vein of the ear, there is an initial drop in particle concentration in blood (Fig. 3). Ten to fifteen minutes after injection the animals usually show an asthmatic breathing, and, if the animals are killed during that period, as much as 40 per cent of the injected dose may be found in the lungs (Juhlin, unpublished). The particles are then gradually released into the blood, and their concentration in blood increases. The circulation and oxygenation of some of these animals is probably impaired, which might cause the slower bile secretion and thus also decrease the excretion per minute in bile.

Thanks are due to Mrs. Asta Palis for valuable technical assistance and to Dr. B. af Ekenstam and Mr. A. Furendal, A.-B. Nobelkrut, Bofors, Sweden, for preparation of the particles.

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Inhibitory Effects of Hydrochloric Acid in Antrum and Duodenum on Gastric Secretory Responses to Test Meal in Pavlov and Heidenhain Pouch Dogs

By

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Abstract

ANDERSSON, S. *Inhibitory effects of hydrochloric acid in antrum and duodenum on gastric secretory responses to test meal in Pavlov and Heidenhain pouch dogs.* Acta physiol. scand. 1960. *49.* 231—241. — Instillation of hydrochloric acid into the excluded duodenum inhibits the gastric secretion stimulated by test meal in both Pavlov and Heidenhain pouch dogs. This is in accordance with the assumption by several authors that a secretion-inhibiting mechanism is activated by an acid milieu in the duodenum. The results also show that this mechanism operates independent of the vagal nerve supply to the gastric mucosa. Perfusion of an isolated antral pouch with hydrochloric acid had lesser and irregularly occurring inhibitory effects on the secretory responses in Pavlov pouch dogs.

In a previous paper (ANDERSSON 1960) it has been shown that the fasting secretion occurring in Pavlov and Heidenhain pouch dogs after the exclusion of antrum-duodenum can be inhibited by the instillation of acid into these regions. No definite conclusions as to the more precise nature of the inhibitory mechanisms could be drawn from the observations, one cause of this inability being that the reason for the occurrence of the fasting secretion was obscure. It has therefore been considered of interest to extend the study to the action of the antral-duodenal inhibitory mechanisms upon secretion in-

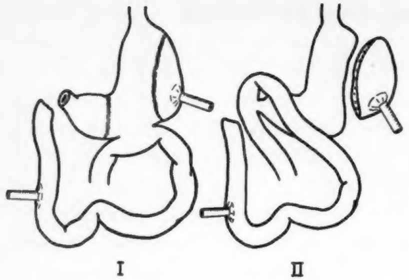

Fig. 1. Operative preparation of the dogs: I. (Dogs 75, 126 and 137) Pavlov pouch; mucosal wall between the corpus and antrum; gastrojejunostomy; antral fistula and cannula in the duodenum.
II. (Dogs 129, 132, 136 and 143) Heidenhain pouch; pylorojejunostomy and cannula in the duodenum

duced by better defined secretory stimuli. In the present series of experiments the inhibition of gastric secretion induced by test meals has been observed.

Method

Seven adult mongrel dogs weighing from 15 to 20 kg were used for the experiments. In three of the animals Pavlov pouches were made (no. 75, 126 and 137). Three to four weeks after this operation antrum-duodenum exclusion was performed as follows. The mucosa was evaginated through a 4 to 6 cm incision (to become the gastrojejunal stoma) along the greater curvature of the stomach as near as possible to the partition between the stomach and the Pavlov pouch. With a circular incision the mucosa was divided at the border of the antrum and corpus; the mucosal margins were dissected out and a strip of mucosa about 1 cm wide was resected from both edges before they were sutured. The resection was performed in order to eliminate as far as possible those parts that were assumed to consist of a mixture of antral and corpus mucosa. The first jejunal loop was then anastomosed with the stomach. In a third operation the pylorus was divided. The duodenal end was closed and the antral end brought out through the abdominal wall as a cutaneous fistula. Finally, a steel cannula was inserted into the duodenum about 10 cm distal to its upper invaginated end (Fig. 1: I).

Heidenhain pouches were prepared in the 4 remaining animals (no. 129, 132, 136 and 143). It had previously on repeated occasions been observed that in Heidenhain pouch dogs with excluded antrum-duodenum or isolated antrum and excluded duodenum the secretory responses to a test meal were slight and uncertain. In the dogs used here, therefore, the antrum was retained in continuity with the stomach and the animals were prepared in a second operation as follows. The pylorus was transected and the duodenum closed, while a new gastrointestinal passage was created through an anastomosis between the divided pylorus and the first loop of the jejunum. Access to the duodenum was attained, as in the Pavlov pouch dogs, by the introduction of a cannula (Fig. 1: II).

All operations were performed under pentobarbital sodium anesthesia (25—40 mg/kg intravenously). For the postoperative management and subsequent care of the dogs see Uvnäs *et al.* (1956).

Between operations and before the experiments were begun the animals were allowed a period of at least three weeks for recovery.

When the experiments were started in the morning the animals had been fasting for 18 to 24 hours. During at least one hour before feeding the fasting secretion was

Table 1. Hourly secretory responses to test meal in Pavlov pouch dogs with and without concomitant instillation of HCl into the duodenum and antrum

A) Dog no. 75. Test meal: 50 g meat.

		Secretion (meq total acid)				
		Control period (one hour)	After test meal			
	Exp. no.		1st hr	2nd hr		3rd hr
Controls	1	0.04	2.58	2.29	1.99	2.06
	2	0.06	2.43	1.53	1.15	2.15
	3	0.01	2.90	2.09	1.68	2.37
	4	0	2.05	1.61	1.45	2.63
	Mean	0.03	2.49	1.88	1.57	2.30
N/5 HCl in duodenum	1	0.20	**0.53**	**0.40**	0.32	2.42
during the 1st and	2	0.18	**0.16**	**0.04**	0.80	3.28
half the 2nd hours	3	0.15	**0.29**	**0.25**	0.68	2.74
	Mean	0.18	**0.33**	**0.23**	0.60	2.81
Mean percentage of controls		—	**13**	**12**	38	122
N/10 HCl in antrum	1	0.03	**2.63**	**2.05**	1.50	1.73
during the 1st and	2	0.49	**3.38**	**1.37**	1.40	1.88
half the 2nd hours	3	0.37	**2.63**	**1.25**	1.26	2.87
	4	0.05	**2.03**	**0.87**	0.72	1.79
	Mean	0.24	**2.67**	**1.39**	1.22	2.07
Mean percentage of controls		—	**107**	**74**	78	90

B) Dog no. 126. Test meal: 50 g meat.

		Secretion (meq total acid)				
		Control period (one hour)	After test meal			
	Exp. no.		1st hr	2nd hr	3rd hr	4th hr
Controls	1	0.07	1.91	1.80	1.26	1.01
	2	0.33	1.91	2.07	1.31	0.94
	3	0.17	1.78	1.48	1.06	0.49
	Mean	0.19	1.87	1.78	1.21	0.81
N/5 HCl in duodenum	1	0.13	**0.13**	1.72	1.48	0.45
during the 1st hour	2	0.12	**0.17**	1.70	1.24	0.52
	3	0.05	**0.11**	1.68	0.87	0.28
	Mean	0.10	**0.14**	1.70	1.20	0.42
Mean percentage of controls		—	**8**	96	99	52
N/10 HCl antrum	1	0.07	**1.66**	1.35	0.66	0.27
during the 1st hour	2	0.21	**1.23**	1.00	0.46	0.24
	3	0.20	**1.46**	1.17	0.44	0.29
	Mean	0.16	**1.45**	1.17	0.52	0.27
Mean percentage of controls		—	**78**	66	43	33

Table 1 (Cont.)

C) Dog no. 137. Test meal: 250 g meat.

		Secretion (meq total acid)				
		Control period (one hour)	After test meal			
	Exp. no.		1st hr	2nd hr	3rd hr	4th hr
Controls	1	0	1.58	1.66	1.00	0.66
	2	0.02	1.58	1.81	0.89	0.46
	3	0	1.40	1.67	0.95	0.70
	Mean	0.01	1.52	1.71	0.95	0.61
N/5 HCl in duodenum during the 1st hour	1	0	**0.34**	1.79	1.16	0.74
	2	0	**0.07**	0.37	0.65	0.47
	3	0	**0.13**	0.63	0.53	0.43
	4	0	**0.16**	1.37	1.24	0.71
	Mean	0	**0.18**	1.04	0.90	0.59
Mean percentage of controls		—	**12**	61	95	97
N/10 HCl in antrum during the 1st hour	1	0	**1.37**	1.36	0.50	0.22
	2	0	**1.59**	1.25	0.76	0.39
	3	0	**1.73**	1.11	0.33	0.21
	4	0	**1.41**	1.41	1.01	0.68
	Mean	0	**1.53**	1.28	0.65	0.38
Mean percentage of controls		—	**101**	75	68	62

recorded. The secretory response to the test meal was then followed for 3 to 4 hours. The secretory responses varied from animal to animal, and the amount and composition of the test meal had therefore to be adjusted to the secretory pattern of each animal. The Pavlov pouch dogs were fed a single dose of 50 to 250 g boiled and ground lean meat, the largest amount being given to animals with a poor secretory response. In the Heidenhain pouch dogs the secretory responses to a test meal were smaller and the course of secretion was more even than in the Pavlov pouch dogs. If one is to obtain clear and significant inhibitory effects, there must be a reasonably good basic secretion. Accordingly, if the secretory response to a single feeding was too low the feeding was repeated up to 5—9 times at intervals of 15 min. Dog no. 136, whose secretion was poor, was given a test meal consisting of a mixture of meat and bone dust. Such a mixture has been shown (Öbrink 1954) to constitute a strong secretory stimulus. The mode of administration and the composition of the test meals for the different animals are shown in Table I and II. Despite this variation in the feeding procedure the experimental results in the different animals were in agreement.

The gastric secretory output was collected in 15-min portions. The volume was measured, and the quantities of free and total acid were determined by the titration of each portion with N/100 NaOH, Töpfer's reagent and phenolphthalein being used as indicators.

In experiments in which HCl was instilled into the duodenum a double-lumen rubber catheter was introduced through the duodenal cannula. With the help of a guide the point of the catheter was pushed up as far as possible into the upper part

Table II. Hourly secretory responses to test meal in Heidenhain pouch dogs with and without concomitant instillation of HCl into the duodenum.

A) Dog no. 129. Test meal: 50 g meat.

		Secretion (meq total acid)				
		Control period (one hour)	After test meal			
	Exp. no.		1st hr	2nd hr	3rd hr	4th hr
Controls	1	0.27	1.02	1.48	1.12	0.69
	2	0.16	0.86	0.87	0.66	1.17
	3	0.10	0.64	0.62	0.43	0.53
	Mean	0.18	0.84	0.99	0.74	0.80
N/5 HCl in duodenum during the 1st hour	1	0.18	**0.09**	1.16	0.98	0.71
	2	0.10	**0.05**	1.81	1.17	0.91
	3	0.07	**0.11**	0.63	0.56	0.46
	4	0.10	**0.11**	0.54	0.50	0.50
	Mean	0.11	**0.09**	1.04	0.80	0.65
Mean percentage of controls		—	**11**	105	107	81

B) Dog no. 132. Test meal: 50 g meat every 15 minutes during the 1st hour.

		Secretion (meq total acid)				
		Control period (one hour)	After test meal			
	Exp. no.		1st hr	2nd hr	3rd hr	4th hr
Controls	1	0.22	1.15	1.36	1.15	1.28
	2	0.10	0.94	1.18	1.26	0.92
	3	0.20	0.59	0.80	0.66	0.73
	4	0.15	0.73	0.78	0.83	0.80
	5	0.39	1.55	1.27	1.66	1.77
	Mean	0.21	0.99	1.08	1.11	1.10
N/5 HCl in duodenum during the 1st hour	1	0.05	**0.36**	0.80	1.25	1.00
	2	0.30	**0.16**	0.43	0.46	0.46
	3	0.06	**0.17**	0.72	0.46	0.52
	4	0.26	**0.14**	0.36	0.41	0.50
	5	0.31	**0.23**	0.44	0.78	0.55
	Mean	0.20	**0.21**	0.55	0.67	0.61
Mean percentage of controls		—	**21**	51	60	55
N/5 HCl in duodenum during the 2nd hour	1	0.07	0.76	**0.30**	0.83	1.01
	2	0.31	1.36	**0.86**	2.03	1.24
	3	0.16	0.69	**0.20**	0.71	0.71
	4	0.27	0.88	**0.29**	0.67	1.71
	5	0.15	1.61	**0.88**	1.37	2.17
	Mean	0.19	1.06	**0.51**	1.32	1.37
Mean percentage of controls		—	107	**47**	119	125

Table II (Cont.)

C) Dog no. 136. Test meal: 50 g bonedust-meat mixture every 15 minutes during the 1st and 2nd hours (30 g bonedust per 100 g meat)

		Secretion (meq total acid)				
		Control period (one hour)	After test meal			
	Exp. no.		1st hr	2nd hr	3rd hr	4th hr
Controls	1	0.08	0.56	1.80	2.47	2.32
	2	0.03	0.10	1.68	2.56	2.48
	3	0.07	0.32	1.96	2.72	2.65
	Mean	0.06	0.33	1.81	2.58	2.48
N/5 in duodenum	1	0.04	**0.14**	**0.58**	2.69	2.33
during the 1st and	2	0.04	**0.06**	**0.57**	2.38	2.94
2nd hours	3	0.10	**0.30**	**0.68**	2.67	2.34
	Mean	0.06	**0.17**	**0.61**	2.58	2.54
Mean percentage of controls		—	**52**	**34**	100	102

D) Dog no. 143. Test meal: 250 g meat.

		Secretion (meq total acid)				
		Control period (one hour)	After test meal			
	Exp. no.		1st hr	2nd hr	3rd hr	4th hr
Controls	1	0.09	1.56	0.56	0.55	0.79
	2	0.03	0.67	0.71	0.51	0.75
	3	0 09	0.98	0.74	0.35	0.31
	4	0.62	1.28	0.44	0.55	1.01
	Mean	0.21	1.12	0.61	0.49	0.72
N/5 HCl in duodenum	1	0.02	**0.33**	0.95	0.44	0.67
during the 1st hour	2	0.19	**0.21**	0.65	0.81	0.97
	3	0.19	**0.22**	1.71	1.00	0.81
	4	0.18	**0.36**	0.86	0.57	0.84
	Mean	0.15	**0.28**	1.04	0.71	0.82
Mean percentage of controls		—	**25**	171	145	114

of the duodenum, *i. e.* close to the upper invaginated end of the latter. Samples of the duodenal content (15—25 ml) were collected for pH determination; these were taken some centimeters below the place of entry of the acid every 15 min during the course of acid infusion. In order to exclude the possibility of infused acid reaching antrum (which in the Heidenhain pouch dogs was left in situ, Fig. 1: II), samples for pH determination were taken at the site for pylorojejunostomy. Neither when HCl nor NaCl were instilled into duodenum was there any appreciable difference in the pH of such intestinal samples.

Acid perfusion of the antrum was carried out in a similar manner, rubber catheters

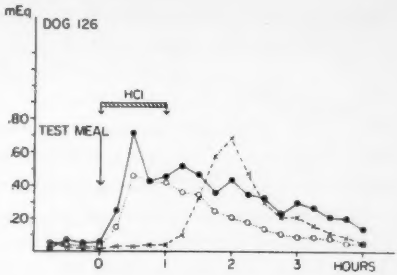

Fig. 2. Inhibition of secretory response to test meal in a Pavlov pouch dog by instillation of HCl into the duodenum and antrum. ●———●: Control (3 exp.); ×———×: N/5 HCl in duodenum (3 exp.); ○-----○: N/10 HCl in antrum (3 exp.).

being used. Special care was taken not to increase the pressure in the antral pouch during the perfusion. For acid perfusion of the antrum N/10 HCl was used. Repeated pH determinations carried out on the perfusate showed that there was only a very slight rise in the pH of the perfused solution with the perfusion rates employed, *i. e.* about 100 ml per hour. The pH of the perfusate never exceeded 1.5.

In the majority of the experiments the instillation of acid into the antrum or duodenum was started concomitantly with the feeding. The instillation was maintained for 1—2 hours. In some experiments the instillation was not started until one hour after the feeding, and in these cases it was maintained for one hour.

During the duodenal instillation of acid the quantity and concentration of the infused acid were adjusted in such a way as to keep the duodenal pH at about 2. In this connection it was found that when N/10 HCl was instilled the duodenal pH oscillated 3 to 4 units during the infusion period, and this despite the constant instillation of relatively large volumes of acid. On the other hand, it was possible to maintain a constant low pH when considerably smaller quantities (about 100 ml per hour) of stronger acid (N/5) were used. The stronger acid was accordingly used throughout in these experiments.

Results

The mean secretory response to feeding in a Pavlov pouch dog is exemplified in Fig. 2. The instillation of hydrochloric acid into the excluded duodenum reduced the secretory response during the first postprandial hour by, on an average, 90 per cent. As has already been mentioned, the pH in the duodenum during the instillation period was checked at regular intervals, and was in all experiments adjusted to approximately pH 2. When the acid instillation was stopped the intraduodenal pH rose and the secretion increased concurrently to the control level or, in many cases, exceeded this. Table I gives the mean secretion per hour with (10 exp.) and without (10 exp.) acid instillation in the different animals.

Perfusion of the isolated antral pouch with hydrochloric acid seemed to have a slight effect on the secretion. A typical secretory response with antral acid perfusion is shown in Fig. 2. As may be seen from the figure, there was no appreciable depression of the secretory response during the perfusion period.

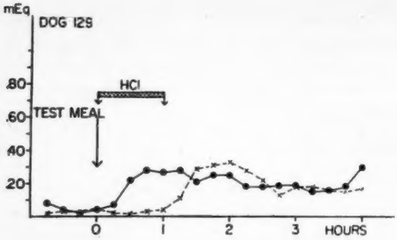

Fig. 3. Inhibition of secretory response to test meal in a Heidenhain pouch dog by instillation of HCl into the duodenum. ●——●: Control (3 exp.); ×---×: N/5 HCl in duodenum (4 exp.).

In 11 such experiments on the 3 animals the same behaviour was observed. The secretory responses during antral acid perfusion amounted to 107, 78 and 101 per cent respectively of the controls (Table I). During the later part of the secretory period, on the other hand, when the acid perfusion had been discontinued, a moderate reduction was often observed. As may be seen from Table I, during the period from the second to the fourth hour the mean level of secretion tended to fall below that for the controls.

The mean secretory response to a test meal in a Heidenhain pouch dog is shown in Fig. 3. When the intraduodenal pH was depressed to about 2 the secretion was almost totally inhibited. Similar results were obtained in 16 such experiments on four animals (Table II).

In those experiments on both Pavlov and Heidenhain pouch dogs in which on account of insufficient acid administration the duodenal pH remained at approximately 5—6 or else varied widely, the inhibitory effect obtained was either negligible or entirely absent.

In the experiments described above the inhibitory effect of acid in duodenum was studied on the initial part of the secretory responses. In 5 experiments on dog no. 132, on the other hand, the intraduodenal instillation of acid was not started until the secretion was well developed. The results are summarized in Table II B. As may be seen, the introduction of acid reduced the secretion with about 50 per cent compared with controls.

There was no difference between the results if the secretion was expressed in mEq free HCl instead of in mEq total acid as in Table I and II.

Discussion

Recently ANDERSSON (1960) has shown that the instillation of HCl into excluded antrum-duodenum effectively inhibits a fasting secretion in Pavlov and Heidenhain pouch dogs. The observation was taken to indicate the existence of an intraduodenal pH-sensitive inhibitory mechanism, humoral in character. This latter presumption was based on the observation that the secretion was inhibited just as effectively in a vagally denervated as in a vagally innervated

pouch. The present results support the above assumption. The gastric secretory response to a test meal is inhibited in both Heidenhain and Pavlov pouch dogs when the intraduodenal pH is reduced to about 2.

Earlier observations concerning the intimate nature of the duodenal inhibitory mechanism contradict one another. Some writers (*e. g.* CODE and WATKINSON 1955, SIRCUS 1958) consider that it is a matter of a nervous mechanism, whereas the experiments of others (PINCUS *et al.* 1944, GREENLEE *et al.* 1957) tend rather to support a humoral mechanism. It is difficult to give any satisfactory explanation of the discrepancies between the results of different writers. In the light of the fact that the HCl secretion in the stomach is the result of a balanced interplay between secretion-stimulating and secretion-inhibiting gastroduodenal mechanisms it may be assumed that the surgical preparation of the animals used in the experiments strongly influences the results arrived at by different investigators. CODE and WATKINSON (1955), for example, used dogs with intact antral-duodenal passage. Consequently, acid gastric contents must have passed down into the duodenum, and the duodenal inhibitory mechanism must be assumed to have been active even in their control experiments. In the animals used in the present investigation, on the other hand, the duodenum was surgically excluded from contact with acid gastric contents. The inhibitory mechanism was excluded, and any inhibitory effect elicited by intraduodenal changes in pH should here be more easily detected.

Fat is believed to exercise its inhibitory effect on the gastric secretion via a humoral duodenal mechanism. The inhibition elicited through the instillation of hypertonic saline and sugar solutions into the duodenum is presumed to take place in a similar manner. The pH-sensitive duodenal inhibitory mechanism appears to act with equal effectiveness on secretion from a Heidenhain or a Pavlov pouch. It is therefore reasonable to assume that a similar humoral mechanism may be brought into play when the secretion is inhibited by an acid pH in the duodenum, *i. e.* that the acid releases an inhibiting substance. The possibility that the inhibitory effect is mediated via sympathetic nerves cannot, however, be completely excluded. The significance of such nerves for the control of gastric secretion is, though, entirely unknown. DRAGSTEDT *et al.* (1951) studied the effect of sympathectomy on the gastric secretion in dogs, but found no change in the secretory pattern of a vagally denervated stomach after this operation. Nor were FORREST and CODE (1954) able to demonstrate any change in the secretory behaviour in Pavlov and Heidenhain pouches after "postganglionic sympathectomy". It is therefore not very likely that an intact sympathetic innervation to the stomach constitutes a prerequisite for the observed inhibition.

Secretory inhibition triggered off by reduction of intraduodenal pH may, however, also be conceived to take place via inhibition of gastrin release from the antral mucous membrane, *e. g.* through nervous inhibitory reflexes

from the duodenum to the antrum. But there is no evidence to show that this is the case.

Acid perfusion of the antral pouch in the Pavlov pouch dogs did not cause any appreciable inhibition of the secretory responses during the period for which the perfusion lasted. This was rather surprising, as several writers (Margolus and Harrison 1957, Gouws and Harrison 1958, State and Morgenstern 1958) have found that acid perfusion of an isolated antral pouch inhibits the secretory responses to a test meal almost totally in Heidenhain pouch dogs and by more than 50 per cent in Pavlov pouch dogs. It is hard to say to what these differences are due, but one reason might be that these writers perfused the antrum throughout the whole period for which the secretory response lasted, *i. e.* for 4—6 hours. The most essential difference between these investigations and the present one, however, is that the isolated "antral" pouch in their dogs appears to have comprised a part of the upper duodenum. Concerning the preparation of the isolated antral pouch, Guows and Harrison (1957), for example, state that the "duodenum was divided *distal* to pylorus, which was brought out as a cutaneous fistula". The significance of this is, however, uncertain; but if the duodenal inhibitory mechanism, as suggested by Shay, Gershon-Cohen and Fels (1942), involves also the upper duodenum just distal to pylorus, the duodenal inhibitory mechanism may have been activated in their experiments. The present results do not seem to permit of any fruitful discussion about the antral inhibitory mechanism and its functional significance.

Conclusions

The instillation of HCl into the duodenum effectively inhibits the gastric secretory response to a test meal in as well Pavlov as Heidenhain pouch dogs. This favours the assumption that the duodenal pH-sensitive inhibitory mechanism acts humorally rather than via nerve channels. It is, however, not yet possible definitely to exclude other alternatives. Perfusion of the isolated antrum with HCl produced considerably smaller and more irregular inhibitory effects upon the secretory responses from Pavlov pouches. The significance of this antral inhibition is doubtful.

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The Determination in Vivo of the Rate of Ciliary Beat in the Trachea

By

TORE DALHAMN

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Abstract

DALHAMN, T. *The determination in vivo of the rate of ciliary beat in the trachea.* Acta physiol. scand. 1960. *49.* 242—250. — The rate of ciliary beat in the trachea of live rabbits was recorded by filming at 220 frames per second. The animals were able to breathe through their normal respiratory tracts, by a special connection between the microscope and the trachea. The mean rate from 31 subjects was 1,099 beats per minute, the deviation between the animals 172 beats per minute, and the error of method 40 beats per minute. The rate of beat was correlated to the rectal temperature, but did not show any variation during the two hours the experiments lasted. The method can be used for investigations into the effects of inhaled agents (gases, particles) on the ciliary activity of the trachea.

In recent years discussion on air pollution and its biological effects on the human body has become increasingly vivid. Two problems have come to the fore in this discussion, namely the effects of chronic exposure, such as *e. g.* the carcinogenic effect (HUEPER 1955, KOTIN 1956), and the effect of a more acute influence, such as that statistically demonstrated in an increase of the death rate during periods of strongly increased air pollution — smog (LOGAN 1953, 1956). It should be pointed out that the mucosa of the respiratory tracts, which is among the first tissue to come into contact with the inhaled agent, reacts with both morphological and functional changes.

Morphological changes caused by the inhalation of pulmonary-irritant gases and mists, and also by tobacco smoke have previously been demonstrated (AMDUR 1952, DALHAMN 1956, and LEUCHTENBERGER 1958). Functional

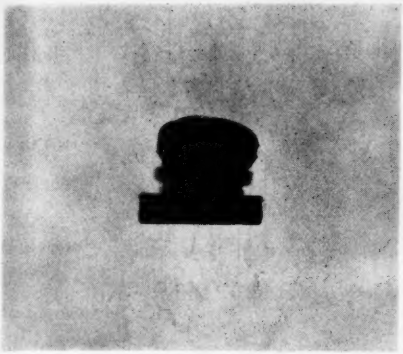

Fig. 1. The rubber bellows connected to the trachea and the microscope.

changes after the inhalation of sulphur dioxide have been studied, in connection with rate of breathing, by AMDUR (1953) and others, and, as regards the effect on the ciliary movement and mucous transport, by DALHAMN and RHODIN (1956).

With the method evolved by DALHAMN (1956), the tested gases, warmed and moistened, were blown over the exposed trachea. If, however, one wants to obtain a more physiological picture of the course of events in the mucosa on exposure to *e. g.* pulmonary-irritant gases, and, particularly, in discussion of the maximum allowable concentration, it would seem to be an advantage if the substances in question can be introduced via the normal respiratory channels, and in such a manner that the animal itself inhales them. By the present method it is possible to study the effects of different substances on the ciliary activity in the trachea of animals, the substances being inhaled by the animals themselves through the normal respiratory tracts.

Method

As regards the recording of the ciliary beat, the method does not involve any change in principle from a method previously published (DALHAMN 1955, 1956). No determination of the mucous flow has been attempted in the present case (DALHAMN 1956).

A film camera was used for the registration of the ciliary beat, which was calculated from the exposed film as projected afterwards. Each determination of the rate of beat is based on three different estimations from the same film. In order to avoid as far as possible any subjective influence during the estimation, the three parts have not been estimated in immediate succession, but have been chosen arbitrarily and in connection with the estimation of other films.

In order to test the individual variations in the estimation of the films, two female laboratory workers M. L. and I. P. were given the job of determining, independently of each other, the rate of beat in 10 different films not included in the material reported below. These were of previously filmed trachea in rats. The results showed practically identical mean figures: 1,027 and 1,029 beats per minute respectively. The error of

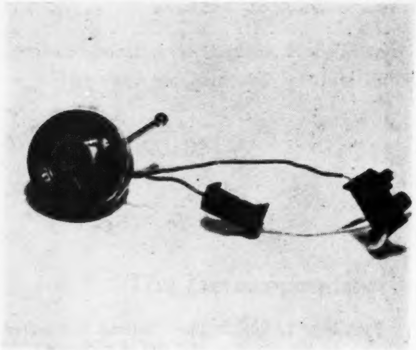

Fig. 2. The lens of the microscope fitted with the heating coil.

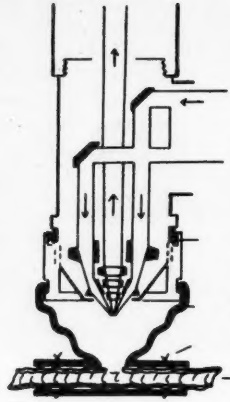

Fig. 3.
Schematic drawing of the apparatus.

method in the calculations was measured as 27 and 28 beats per minute respectively, *i. e.* less than 3 per cent of the mean. The estimations of the inter-animal deviation were also very much in agreement, namely 164 and 175 beats per minute.

The microscope used was the same as in previous experiments *i. e.* a Leitz "Ultrapak" for vertical light, with a 5 × ocular and a 6.5 objective. Light was provided by a Philips sound film lamp type 6019 U, for 8 V and 6 amp. In order to prevent the warming of the trachea while the microscope was being used an interference filter (Filtroflex type B) was placed in the path of the beam. The film camera used was a Bell and Howell Traide 200, the same model as used previously (Dalhamn 1959), with a speed of 220 exposures per sec. The connection between the microscope and the trachea was arranged as follows. A rubber bellows, as shown in Fig. 1, was a) fitted round the lens of the microscope, and b) made to embrace the trachea via a piece of rubber tubing that was slit along its length and secured to the bellows. At the top of the bellows, where it was secured to the tubing, there was a hole sufficiently large to permit observation through the microscope.

Since the respiratory system from the nasopharynx via the trachea to the lungs must permit the passage of air only through the nasopharynx, the rubber bellows must fit the lens perfectly, while the lens itself must have an airtight connection with the condenser, which with this type of lens is built in with the lens. The airtight connection with the lens has been made by adapting the largest diameter of the bellows to that of the lens, while the lens was made airtight by filling the circular interval between the lens itself and the condenser with plastic. In order to prevent any condensation on the lens when this comes into contact with the warm, moist air of the trachea, the lens has been fitted with a heating coil that keeps it at a constant temperature as required (Fig. 2). Finally, an airtight connection of the rubber tubing round the trachea was secured by a suture round the trachea and the tubing (Fig. 3). The material of the bellows was so chosen as not to permit expansion and contraction in time with the respiratory phases of the animal, as this would have meant the introduction of "dead space". The material was soft enough, however, to permit movement, and, thus, the focussing of the microscope.

The trachea was very carefully dissected free before the tracheotomy, which was then performed in the following way. After the burning of a small hole in the trachea by

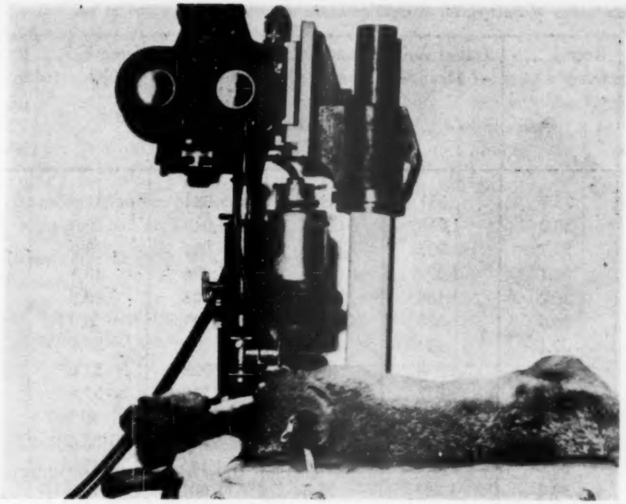

Fig. 4. The apparatus assembled.

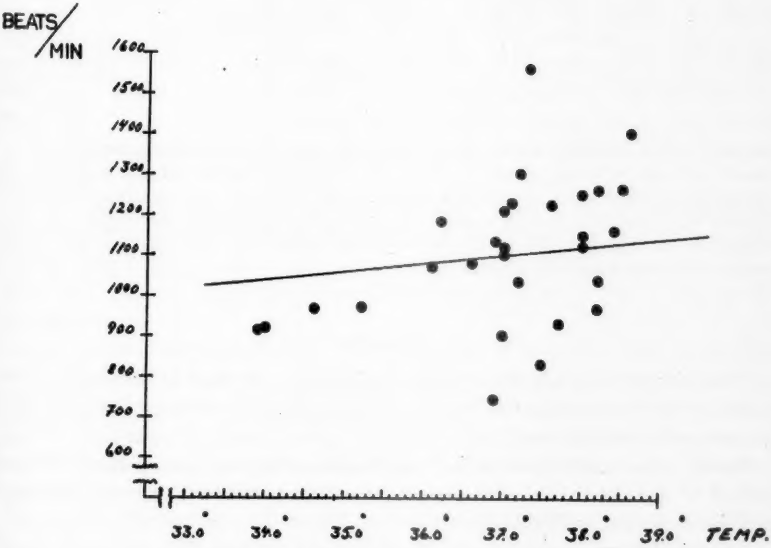

Fig. 5. Ciliary beat frequency in relation to rectal temperature in the rabbit.

Table II. Influence of time factor on rate of beat

Rabbit no.	Rectal temp. °C	Initial value — Mean —	Rectal temp. °C	Rate of beat in beats per minute after 1 hour. Mean	Rectal temp. °C	Rate of beat in beat per minute after 2 hours. Mean
1	33.9	911	33.9	988	31.0	1,132
2	38.0	1,255	38.0	1,049	38.0	1,161
3	37.7	937	37.1	894	38.0	1,151
4	36.1	1,072	35.5	1,096	35.4	1,267
5	36.2	1,186	36.6	1,107	36.8	1,168
6	36.6	1,083	36.1	1,449	36,0	1,225
7	37.1	1,231	37.4	1,180	38.2	1,284
8	37.2	1,038	38.0	799	37.6	889
9	38.2	972	38.2	779	37.9	836
10	37.0	904	36.7	1,030	36.7	1,030
11	36.9	1,135	36.6	1,257	36.6	1,353
12	38.0	1,126	37.5	1,144	37.5	995
13	38.2	1,267	39.8	901	40.0	928
14	38.6	1,404	38.6	1,523	38.5	1,390
15	37.3	1 561	36.9	1,413	37.1	1,206
16	38.4	1,162	37.0	1,122	37.2	1,146
17	37.2	1,303	37.0	1,206	37.2	1,324
18	37.0	1,123	38.2	1,214	38.2	1,315
19	37.0	1,213	35,8	1,096	35.9	1,122
20	37.6	1,226	37.9	1,090	38.6	1,055
Mean	37.2	1,156	37.1	1,117	37.1	1,149

diathermy, the definitive tracheotomy was performed by the clipping away of the required amount of tissue. Bleeding was checked by etching round the edges of the tracheotomy using weak diathermy. Immediately after the tracheotomy the rubber bellows were placed in position round the trachea and connected to the microscope, after which the recording of the ciliary activity took place (Fig. 4, a and b). The rectal temperature was regularly controlled.

Results

The object of the study was a) to determine a normal value for the rate of beat and b) to find out whether the length of time in which the animal lies in position has any effect on the beat.

Normal data were collected from 31 rabbits, of which 20 were observed for a period of two hours (Table II), while the influence of the time factor on ciliary activity is shown in Table I.

Statistical treatment of the results in Table I showed that the mean figure for ciliary beat in 'normal animals' was 1,099 beats per minute, or about 20

Table I. Comparison of rate of beat, inter-animal deviation, and experimental error, with the use of different types of animal and different methods

Method of investigation	Rate in beats per minute	Inter-animal deviation in beats per minute	Experimental error in beats per minute
Live rats in moist chamber, 128 exposures per second, 37 animals	1,317	176	136
Live rabbits in moist chamber 220 exposures per second, 10 animals	1,099	162	35
Live rabbits, normal channels of respiration, 220 exposures per second, 31 animals.	1,099	172	40

beats per second. By variation analysis 2 components, were separated, one connected with differences between the animals themselves, the other with errors in calculation from the projection of the films (3 calculations per film). The standard deviation as regards variation between the animals was 172 beats per minute, or 15.7 per cent of the mean. The experimental error obtained in the calculation of the rate of beat was 40 beats per minute, or 3.6 per cent of the mean.

In previous investigations the ciliary beat, and, particularly, the mucous flow, have proved to be dependent on temperature variations in the tissues, an increase in temperature leading to an increase, and a decrease in temperature to a decrease of both the rate of ciliary activity and the mucous flow. The range within which the rectal temperatures of 'normal rabbits' varied was between 33.9—38.6° C, with a mean of 36.9 ± 0.2. By correlation analysis it can be shown that the relation between rectal temperature and rate of ciliary beat is significant $(0.01^{xx} > P > 0.001)$.

Table II shows the influence of the time factor on the beat in the given research conditions. The material of these experiments consisted of 20 animals, and the beat rate was recorded a) initially, b) after one hour, and c) two hours after the initial recording. The animals were the same as the first 20 of the 31 in Table I.

The statistical treatment of the results in Table II gives a mean initial beat frequency of 1,156 beats per min. One hour later the corresponding figure was 1,117 beats per min, and after 2 hours it was 1,149. Between these three means there is no statistically demonstrable difference. The standard deviation between animals was 134 beats per minute, and the experimental error in the estimation from the film was 40 beats per minute.

As regards the rectal temperature, the mean at the series of initial recordings was 37.2 (range 33.9—38.6), while the mean one hour later was 37.1 (range 33.9—39.8), and finally, two hours later 37.1 (range 31.0—40.0).

Discussion

The object of work on the method outlined above has been to make it possible to study the ciliary activity in the trachea of live animals when breathing through their normal respiratory tracts while the observations were made.

The mean rate of beat was 1,099 beats per minute, or about 20 beats per second. In previous experiments the ciliary activy has been recorded from the isolated trachea of rabbits, and the mean in 10 animals was 1,032 beats per min. These values are not in disagreement, nor does the rate of ciliary beat in the trachea of live rabbits differ from the values obtained from live rats, with which the writer obtained an average activity of 1,317 beats per minute, 37 animals being used.

The reliability of the means recorded was statistically investigated by determining a) the inter-animal deviation, and b) the error of calculation from the reading-off of the films. In the normal material of 31 rabbits, the inter-animal deviation was 172 beats per minute, and the experimental error 40 beats per minute. If one compares these figures with the inter-animal deviation of 176 beats per minute and the experimental error of 136 beats per minute obtained in the previous in vivo experiments on rats, one finds that the deviation between the animals seems to be practically identical in groups of rats and rabbits. On the other hand a not inconsiderable reduction of the experimental error has been obtained, namely from 136 to 40 beats per minute. This reduction of the experimental error is clearly the effect of the higher rate of exposure in the filming of the rabbits. In an investigation of the beat rate of live rats with filming at 220 exposures per second, 10 animals being used for the experiment, the inter-animal deviation has been determined as 162 beats per minute, and the experimental error, as 35 beats per minute. With this method, then, it is possible to keep the experimental error at around 40 beats per minute.

From a number of previous experiments it is clear that the temperature of the medium surrounding the cilia, the water temperature, for instance, in the case of bevalves, or the temperature of the tissues in the higher animals, plays a not unimportant role for the frequency of beat. The mean rectal temperature in the material described here was 36.9 (range 33.9—38.6). It would have been preferable if the range could have been less wide, but there proved to be certain difficulties involved in this, particularly since it was intended to record the ciliary activity immediately after tracheotomy. The rectal temperature in the case of certain animals was low, in others high. It is probable that this factor could have been evened out if the animals had been allowed to lie for a shorter or a longer time, but this was considered unsuitable, as it would have introduced an uncontrolled time factor into the experiment.

A correlation estimate between the rectal temperature and the ciliary beat shows a clear correlation ($0.01^{xx} > P > 0.001$), a fact that can hardly be considered surprising in view of the similar results obtained previously. This

does, however, mean that the rectal temperature must always be recorded when determining ciliary beat.

If it is going to be possible to use the method for exposure experiments of different kinds, then it is vital that the rates of beat recorded do not alter simply as the result of the time the animals may be lying prepared.

The figures obtained show that no change in activity could be observed during those two hours. The mean value of the rectal temperature was near enough the same on all three occasions. It is thus probable that the animals can lie prepared for at least two hours without changes in the ciliary activity.

As compared with previous methods of studying the ciliary activity of animals in vivo, the method described above offers certain advantages. The primary gain would seem to be the possibility of having the animal breathe through its normal respiratory channels. As is well known, there are here two functions of importance in the case of inhalation of non-polluted air, namely warming and moistening. Both of these functions are of vital importance for the normal functioning of the upper respiratory tracts. The great sensitivity of the mucosa to, inter alia, drying is well known. With the method previously described by the writer, the warming and moistening of the air was achieved by the placing of the animals (rats) in a warmed and moistened chamber. The upper respiratory tracts were completely disconnected from the respiration, which instead took place through the tracheotomy on the trachea, which was opened lengthwise. With the method described above this procedure is avoided, and the upper respiratory tracts of the animal are responsible to the normal extent for the warming and moistening of the air inhaled.

Of great importance is further the filtration effect, in the case of dust, and the resorption of water-soluble gases. With experiments in a moist chamber, as described above, these functions are completely disconnected, while with the present method the conditions will probably not be significantly different from the physiological.

Another advantage is that the procedure described allows the use of larger animals such as rabbits and cats. As regards smaller animals, it would seem to be technically very difficult to apply a device of the type described above round the trachea of *e. g.* rats.

The method described here makes it possible to study the effects of pollution in the air on the health of human beings, of different gases, gaseous mixtures, and dust on the cilia of the trachea, with the retention of both the heating and moistening effect of the nostrils, and their capacity for filtration and resorption.

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A Comparison of Mast-Cell Reactions in the Rat, Hamster and Guinea Pig

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Abstract

BORÉUS, L. O. *A comparison of mast-cell reactions in the rat, hamster and guinea pig.* Acta physiol. scand. 1960. *49.* 251—260. — Parallel *in vitro* experiments on the mesenterial mast cells were carried out in the rat, hamster and guinea pig. Incubation of mesentery in small, rising concentrations (0.1—100 μg/ml) of compound 48/80, lecithinase A and decylamine caused proportional degrees of "disruption" of the rat and hamster mast cells. pH curves and experiments with enzyme inhibitors gave the same results in the rat and hamster and suggested that, in these two species, the disruption caused by compound 48/80 and lecithinase A involves a similar, enzymatic mechanism, whereas the effect of decylamine is non-enzymatic. The guinea-pig mast cells reacted with dissolution and disappearance (but not with the uniform disruption seen in the rat and hamster) upon incubation with decylamine and lecithinase A. However, high doses and a prolonged incubation time was needed for maximal effect and enzyme inhibitors could not block it. It is concluded that the effects on guinea-pig mast cells was a non-enzymatic lysis of the cell membrane. Compound 48/80 was without effect on the mast cells of the guinea-pig. The resistance to hypotonic solutions was about the same in all three species but the "disruption" of the rat and hamster mast cells occurred much faster than the "disappearance" of guinea-pig mast cells. Provided a sufficient reaction time is allowed, this "disappearance" reaction is the best way of evaluating mast-cell reactions in the guinea pig.

The morphological mast-cell response to histamine liberators is reported to be different in the rat and hamster (PARRATT and WEST 1957). The guinea-pig mast cells are resistant to compound 48/80 (MOTA and VUGMAN 1956,

1956 a) but react with a dose-depending degree of dissolution upon antigen administration both *in vitro* (MOTA 1959, BORÉUS and CHAKRAVARTY 1960) and *in vivo* (BORÉUS 1960). These facts suggested the present study, in which the mast-cell reactions were compared in the rat, hamster and guinea pig, using the technique of NORTON (1954). Thus, mesenterial mast cells from these three species were incubated under identical conditions *in vitro* with three histamine liberators, *viz.* compound 48/80, lecithinase A and decylamine, all of which have been shown to "disrupt" rat mast cells *in vitro* in low concentrations (HÖGBERG and UVNÄS 1957, 1960). The dose-effect curves and pH curves for the mast-cell destroying action of these three drugs were established and the mechanism behind the reactions elucidated by means of experiments with enzyme inhibitors.

Methods

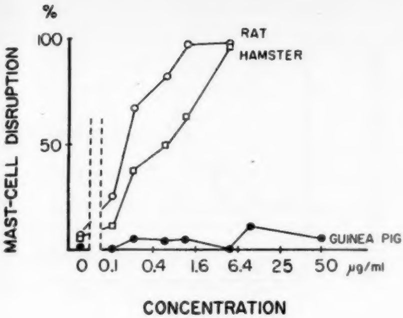
Rats, hamsters and guinea pigs of both sexes were used. They were killed by a blow on the head and bled. Pieces of mesentery were incubated at 37° C in a solution of the following composition: NaCl 9, KCl 0.2, $CaCl_2$ 0.1 g/l. The different histamine liberators or inhibitors were dissolved in this incubation medium. Unless otherwise stated, Sörensen phosphate or barbitone buffer was added in 10 % v/v. If necessary (for producing pH = 10) NaOH was added to the medium. In the inhibition experiments, the mesentery spreads were incubated for 10 minutes with the inhibitor and then placed in the solution of liberator for 10 min (rat and hamster) or 20 min (guinea pig).

After incubation, the mesentery was fixed in 50 % v/v ethyl alcohol with 1 % v/v acetic acid and 4 % w/v lead subacetate for at least 20 min and stained for 30—45 sec in 0.5 % w/v toluidine blue solution in water (pH about 4). Counting was performed at a magnification of 360 ×. 200—300 mast cells, chosen at random, were counted in each specimen and the percentage of "disrupted" cells noted. Any mast cell with 2 or more granules outside the border of the cell was denoted as a "disrupted" cell. When frequency countings were made (in the guinea-pig experiments) the total number of recognizable mast cells in 20 visual fields, chosen at random, were estimated in each specimen. All mast cells, normal as well as morphologically changed, were counted. The term "mast-cell disappearance" means the per cent decrease in cell population as compared with the corresponding control specimen (from the same individual animal). These control specimens were incubated under identical conditions as the test specimens, except that no liberator was present in the incubation medium.

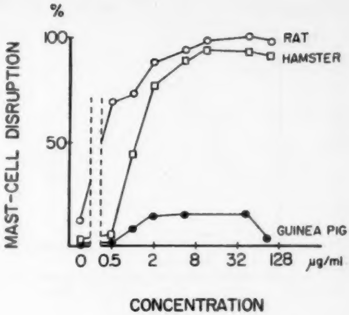
Lecithinase A (from bee venom) was prepared and kindly supplied by Dr. B. Högberg, AB Leo, Hälsingborg, Sweden.

Results

Histamine liberators in rat, hamster and guinea pig. The effect of compound 48/80, lecithinase A and decylamine on the mast-cell "disruption" (as defined above) in the rat, hamster and guinea pig for different doses and at different pH is showed in Fig. 1—2. It is seen that increasing doses of all three liberators gave proportional degrees of "disruption" in the rat and hamster. In both species, the effects of compound 48/80 had a pH optimum at 7.5—7.7. A less

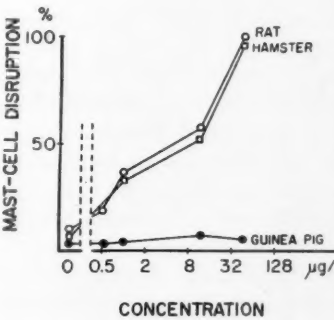


(a)



(b)

Fig. 1. Influence of concentration of compound 48/80 (a), lecithinase A (b) and decylamine (c) on mast-cell disruption. Incubation time 10 min, pH 7.5. Mean values of counts in 4—6 specimens.

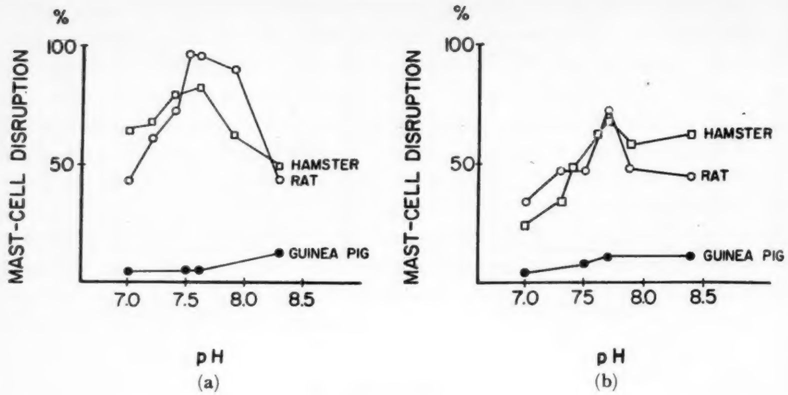


(c)

evident maximum was found for lecithinase A at pH 7.7, whereas no definite pH optimum was obtained with decylamine.

The morphological appearance of the "disrupted" mast cells was the same in the rat and hamster for all three liberators. The cytoplasmic granules were found to be scattered around the cell remnants. The "non-disrupted" cells looked normal and the granules in the cytoplasm seemed to have their normal staining properties.

The guinea-pig mast cells did not show any typical "disruption" following incubation with any of the three liberators. However, lecithinase A and decylamine caused another type of reaction in this species. Several cells showed a coarse fragmentation of the cell body, but only few had reacted with the characteristic "disruption" seen in the rat and hamster. In some cases, there was no change in the outline of the cell but only a subsequent decrease in the metachromatic stainability. Such cells could not be classified as "disrupted". Thus, counting of "disrupted" cells was not a suitable means for evaluating mast-cell reactions in the guinea pig. Therefore, a series of experiments were



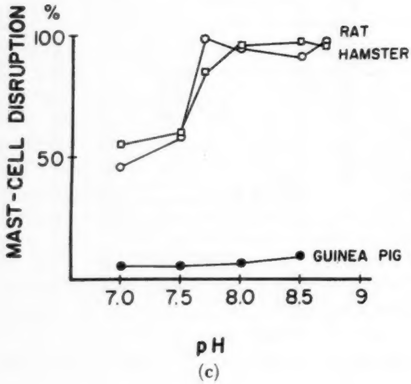


Fig. 2. Influence of pH on the mast-cell disrupting action of compound 48/80 (1 μg/ml) (a), lecithinase A (1 μg/ml) (b) and decylamine (10 μg/ml) (c). Incubation time 10 min. Mean values o counts in 4—6 specimens.

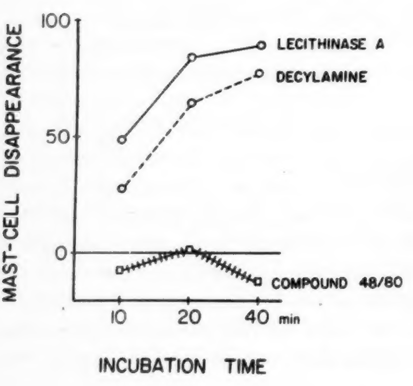


Fig. 3. Influence of incubation time on the mast-cell disappearance from guinea-pig mesentery. Concentrations of liberators 100 μg/ml, pH 7.5. Mean values o counts in 8—12 specimens.

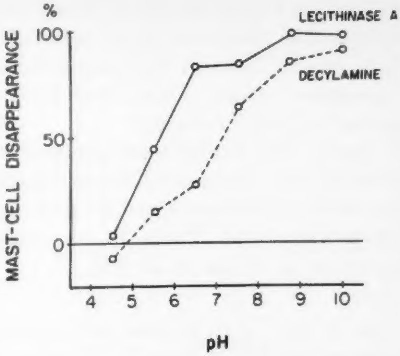


Fig. 4. Influence of pH on the mast-cell disappearance from guinea-pig mesentery. Concentration of liberators 100 μg/ml. Incubation time 20 min. Mean values of counts in 4—7 specimens.

performed, in which were studied the influence of the three liberators on the total mast-cell frequency in mesentery from guinea pigs following incubation at various concentrations and various incubation periods. It was found that lecithinase A and decylamine caused "disappearance" of mast cells, but that maximal reaction required a higher concentration of liberator and a longer incubation time than in the rat and hamster experiments (Fig. 3). On a weight basis, lecithinase A was slightly more active than decylamine. No definite pH optimum was found (Fig. 4) but the reaction was inhibited at low pH. At pH values higher than 10 there was a spontaneous destruction of the mast cells in the controls, whereas no "disappearance" could be demonstrated in the controls at pH values lower than 10. For example, in 5 experiments at pH 7.5, each with 4 mesentery pieces, the mean mast-cell population after 10, 20 and 40 min incubation was 2.40 ± 0.12, 2.46 ± 0.32 and 2.41 ± 0.28 mast cells per visual field, respectively.

Compound 48/80 exerted no demonstrable action on guinea-pig mast cells.

Inhibition of the mast-cell reaction. Mesentery from rat and hamster was incubated in 10^{-3}M ninhydrin or 10^{-3}M ethylmaleimide and then in-

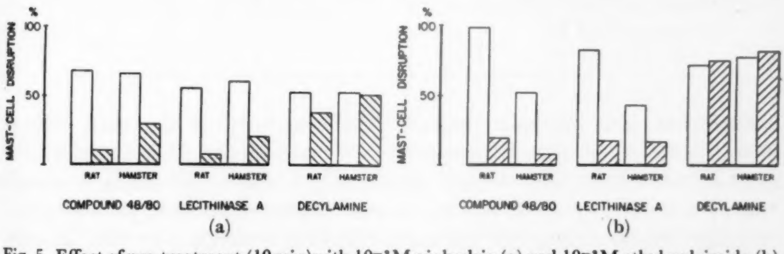


Fig. 5. Effect of pre-treatment (10 min) with 10^{-3}M ninhydrin (a) and 10^{-3}M ethylmaleimide (b) on the mast-cell disrupting action of compound 48/80 (2 μg/ml), lecithinase A (2 μg/ml) and decylamine (20 μg/ml) in the rat and hamster (shaded bars). Empty bars denote corresponding controls (pretreatment in incubation medium without inhibitor). Incubation time with liberator 10 min, pH 7.5. Mean values of counts in 3—6 specimens.

cubated in compound 48/80, lecithinase A or decylamine in order to study any possible inhibition of the mast-cell reaction. The results were similar in both species (Fig. 5). Both drugs caused almost complete inhibition of the "disruptive" action of compound 48/80 and lecithinase A. The effect of decylamine was not influenced.

Similar experiments were made with guinea-pig mast cells. The influence of ninhydrin, ethylmaleimide and iodoacetate on the mast-cell "disappearance" caused by lecithinase A and decylamine was investigated. No inhibition whatsoever was found (Table I), even when the concentration of inhibitor was increased to 10^{-2}M or 10^{-1}M.

Table 1. Effect of pre-treatment with enzyme inhibitors on the mast-cell "disappearance" in the guinea pig caused by lecithinase A (100 μg/ml) and decylamine (100 μg/ml)

Per-cent values as compared to control specimens. Mean values of counts in 3—5 specimens

Incubation procedure		Mast-cell "disappearance"
1 Pre-treatment (10 min)	2 Liberator (20 min)	
Incubation medium	Lecithinase A	93
Ninhydrin 10^{-3}M	»	88
Incubation medium	Decylamine	88
Ninhydrin 10^{-3}M	»	75
Incubation medium	Lecithinase A	76
Ninhydrin 10^{-2}M	»	80
Ninhydrin 10^{-1}M	»	70
Incubation medium	Lecithinase A	98
Ethylmaleimide 10^{-3}M	»	98
Incubation medium	Decylamine	96
Ethylmaleimide 10^{-3}M	»	90
Incubation medium	Lecithinase A	99
Iodoacetate 10^{-3}M	»	90
Incubation medium	Decylamine	91
Iodoacetate 10^{-3}M	»	87

Experiments with hypotonic solutions. The sensitivity of the mast cells to hypotonic solutions was compared in the three species (Fig. 6). When the incubation medium was diluted with distilled water, increasing mast-cell "disruption" was observed in the rat and hamster mast cells, as well as increasing "disappearance" of the mast cells in the guinea pig. The intensity of these reactions were about the same in all three species. Thus, a dilution to 1/16 of the incubation medium gave about 100 % "disruption" in rat and hamster cells and total "disappearance" of guinea-pig cells in 10 min in-

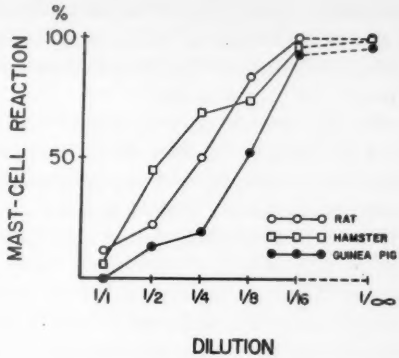


Fig. 6. Effect of dilution of thei ncubation medium with distilled water on the mast-cell reaction, expressed as per-cent disruption in the rat and hamster and per-cent disappearance (from the value for undiluted medium) in the guinea pig. Incubation time 10 min, pH about 7.5. No buffer solution added. Mean values of counts in 4—8 specimens.

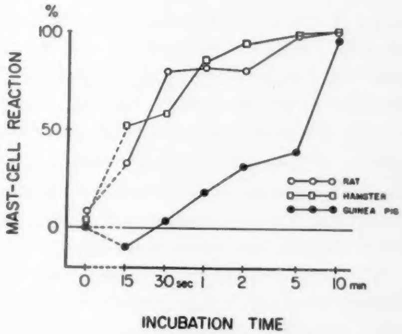


Fig. 7. Influence of incubation time on the mast-cell reaction to distilled water. The reaction is expressed as per-cent disruption in the rat and hamster and per-cent disappearance (from the value for non-incubated specimen) in the guinea pig. pH about 7.5. No buffer solution added. Values for non-incubated specimens at 0. Mean values of counts in 4—8 specimens.

cubation. However, when the time curves for these mast-cell reactions were established (Fig. 7), it was found that the "disruption" was a much faster process than the "disappearance", which reaction consisted in a dissolution of the cells with a gradual loss of the metachromatic material. The "disruption", on the other hand, was rapid; a definite reaction was noted after 15 sec incubation.

Discussion

This paper deals with species differences in mast-cell reaction to histamine liberators and with the histological standardization of these reactions.

It is shown that the mesenterial mast cells in the rat and hamster react upon incubation with three different histamine liberators with the same type of change, *viz.* "disruption", and that the dose-effect curves, pH curves and sensitivity to inhibitors are very similar in these two species. Parratt and West (1957) observed that the disruptive action *in vivo* of compound 48/80

on hamster mast cells in the skin and ears was never more than slight. This was not confirmed in the present experiments *in vitro* on mesenterial mast cells, which showed that small concentrations of the liberator (5 μg/ml) caused almost 100 % disruption.

In their studies on mast-cell disruption *in vitro* in the rat, HÖGBERG and UVNÄS (1960) found that the action of compound 48/80 and, though less distinct, lecithinase A, had a pH optimum around 7.5, whereas the effect of decylamine steadily rose with increasing pH. They also found that various NH_2- and SH-blocking drugs, including ninhydrin and ethylmaleimide, were effective inhibitors of compound 48/80 and lecithinase A but not of decylamine. These results are confirmed in the present study, which also demonstrates similar results in the hamster. The close similarity in the results of all experiments in the rat and hamster in this paper suggests that the mast-cell disruption in these two species involves the same mechanism. The existence of a pH optimum for compound 48/80 and the strong inhibitory effect of the enzyme inhibitors ninhydrin and ethylmaleimide, which block NH_2-groups and SH-groups, respectively, speak in favor of an enzymatic mechanism for disruption in both species. HÖGBERG and UVNÄS (1957) found that lecithinase A was the only enzyme out of 26 which showed a high disruptive action in the rat *in vitro* and suggested that compound 48/80 disrupts the rat mast cells through activation of a lytic enzymatic process hydrolyzing the lipoprotein phospholipids of the mast-cell membrane. Decylamine, on the other hand, seems to act through a non-enzymatic lysis of the cell membrane.

The mast-cell reaction in the guinea pig differed morphologically from the typical, uniform "disruption" seen in the rat and hamster. The fact that "disruption" was only partly demonstrable in the guinea pig does not exclude that a similar discharge of granules from the cell takes place when it reacts to a histamine liberator. It is known that the metachromatic material in the mast-cell granules in the guinea pig (as distinguished from the rat) is very water-soluble. It might be that these granules are dissolved inside or outside the cell during the incubation and that this dissolution makes a demonstration of a "disruption" difficult. It is evident, however, that some reaction, probably at the cell surface, must initiate such a dissolution process with the subsequent disappearance of the cell, because incubation in isotonic salt solution without liberator, even for a considerable time, did not itself lead to a decrease in the mast-cell population. This dissolution seems thus to reflect an initial mast-cell reaction, probably of the same type as the "disruption" in the rat and hamster. It is interesting that the same type of "disruption" of rat mast cells and "disappearance" of guinea-pig mast cells occurs as a result of incubation in antigen (HÖGBERG and UVNÄS 1958, BORÉUS and CHAKRAVARTY 1960). It must be borne in mind that a sufficient reaction time must be allowed to elapse before fixation of the guinea-pig mast cells in order to obtain the full reaction. If this is the case, estimation of the decrease in

mast-cell population is the best way to follow mast-cell reactions in the guinea pig. This method was used for quantitative studies of mast-cell response *in vitro* by MOTA (1959) and BORÉUS and CHAKRAVARTY (1960) as well as *in vivo* by BORÉUS (1960).

It is striking that the effect of lecithinase A on guinea-pig mast cells does not appear until the dose of the drug is elevated and the incubation time prolonged, as compared to the conditions in the rat and hamster experiments. Thus, to get maximal mast-cell "disappearance" in the guinea pig, the dose had to be increased 10 times and the incubation time twice. About the same conditions were found for decylamine. Furthermore, neither the action of lecithinase A nor of decylamine was inhibited by pretreatment of the cells with three different enzyme blocking agents. All these facts suggest that lecithinase A and decylamine do not involve an enzymatic mechanism in their effect on guinea-pig mast cells. It is possible that the enzyme protein complex acts like decylamine in producing a non-enzymatic dissolution of the guinea-pig mast-cell membrane, thereby initiating the lysis of the cell. The specific substrate for the *enzymatic* action of lecithinase A is probably either missing or inaccessible in the guinea-pig mast cells. It is of interest in this connection that the same lecithinase A preparation seemed to have no specific histamine liberating activity in chopped cat skin *in vitro*. Thus, WESTERHOLM (1959), found that 10 and 100 μg/ml of lecithinase A released only 1 μg/g histamine in 30 min incubation, which was about 10 % of the total histamine content. It seems, therefore, that the mast-cell damaging and histamine-releasing effects of lecithinase A varies in different species.

Compound 48/80, even in high doses, gave no mast-cell rseponse in the guinea pig. This confirms the findings of MOTA and VUGMAN (1956 a), who found no histamine liberation from mesentery, skin or lung tissue after injection of this substance and observed no modification in the morphology of the mesenterial mast cells *in vitro* or *in vivo*. Histamine release by compound 48/80 requires much higher concentrations in the guinea pig than in other species. MONGAR and SCHILD (1957) used 100 to 1000 times the concentration normally needed for histamine liberation in cat or rat. FEINBERG and STEINBERGER (1955) concluded that other toxic effects than histamine liberation predominate in the guinea pig, and, in a recent paper, PAPACOSTAS, LOEW and WEST (1959) found that the contribution by released histamine to the lethal effects of compound 48/80 was small. Thus, there is now considerable evidence for the conclusion that compound 48/80 is a weak histamine releaser in the guinea pig, exerting only a slight effect, probably through a non-enzymatic lysis of the mast-cell membrane.

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Circulatory Studies on Substance P in Man

By

HANS DUNÉR and BENGT PERNOW

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Abstract

DUNÉR, H. and B. PERNOW. *Circulatory studies on substance P in man.* Acta physiol. scand. 1960. *49.* 261—266. — Highly purified substance P preparations were infused intravenously in doses of 0.2—1.2 units per kg body weight per min in six healthy subjects. Red flushing, tachycardia and a rapid fall in arterial blood pressure were observed. Cardiac output was slightly increased. The effects were maximal after 1—2 min and gradually decreased during continued infusion.

In the original report by EULER and GADDUM in 1931 substance P was defined as a smooth muscle stimulating and blood pressure depressing factor. The circulatory effect is mainly due to a peripheral vasodilatation (EULER and GADDUM 1931, EULER 1936). In the isolated rabbit's ear HOLTON and HOLTON (1952) observed a vasodilatation following intraarterial administration of substance P. A rapid fall in blood pressure following i. v. injection of substance P is obtained in all mammals and is not blocked by atropine, antihistaminics or ganglionic blocking agents (PERNOW 1953).

In the first study of the effect of substance P in man (LILJEDAHL, MATTSSON and PERNOW 1958) an immediate rise in pulse rate and an immediate fall in arterial blood pressure was observed following intravenous infusion of substance P. A bright red flush was consistently noticed in the face. All subjects felt throbbing sensations in their head. All reactions were transient and disappeared during continued infusion. It was then possible to increase the infusion rate three to four times with no further reactions.

This paper describes further observations on the hemodynamic reactions to continuous intravenous infusion of substance P in man.

Table I. Hemodynamic effects of substance P in five healthy human subjects

Case age in years	Procedure	Pulse rate beats/min	Pressures, mm Hg Right ventricle syst.	end-diast.
1	Before infusion	75	25	5
♂	0.2 U P/kg/min: 1 min	75	26	4
32	5 min	73		
	9 min	74	24	3
	30 min later:			
	0.6 U P/kg/min: 2 min	86	25	3
	6 min	83		
2	Before infusion	76	20	3
♀	0.4 U P/kg/min: 2 min	89	20	4
42	5 min	88		
	After infusion: 2 min	70	16	3
	30 min later:			
	0.6 U P/kg/min: 1 min	114		
	2 min	104	18	4
	5 min	98	16	3
	After infusion: 15 min	78	19	3
3	Before infusion	66	24	5
♀	0.9 U P/kg/min: 2 min	104	26	3
44	5 min	97	25	3
	After infusion: 1 min	72	27	5
	6 min	64	26	5
	30 min later:			
	0.9 U P/kg/min: 2 min	106		
	5 min	103		
4	Before infusion	96		
♂	0.6 U P/kg/min: 2 min	117		
20	6 min	114		
	immediately followed by			
	1.2 U P/kg/min: 2 min	120		
5	Before infusion	66	18	5
♂	0.6 U P/kg/min: 2 min	86	19	4
46	8 min	78		
	immediately followed by			
	1.2 U P/kg/min: 2 min	85	17	5
	8 min	80	18	5

Pulmonary artery			PCV	Brachial artery			Cardiac output	Stroke volume
syst.	diast.	mean	mean	syst.	diast.	mean	l/min	ml
19	8	13	5	132	63	85	8.4	110
19	6	13		124	63	82		
				126	64	88		
17	7	11		128	67	90	8.2	110
14	8	11	6	118	63	85		
16	8	12					9.7	116
19	5	13	6	114	71	88	8.2	108
16	6	12	7	104	68	79		
19	6	13		108	70	81	10.5	118
17	6	13	6	115	70	85		
				88	56	67		
16	6	12		92	58	71		
16	5	11	7	100	67	79	11.8	120
20	6	13		120	73	90	7.8	100
18	8	13	6	126	76	94	6.2	94
15	6	12	5	91	50	66		
20	7	11		107	59	102	8.4	87
19	7	13		137	84	102		
20	8	14		134	79	100	5.7	89
				95	53	68		
				105	57	75		
21	8	15		125	75	98	7.1	74
19	5	12		104	69	82		
16	4	11		106	67	84	8.4	74
16	6	11		108	69	86		
16	6	11	8	119	71	93	6.4	97
15	6	12		100	61	80		
17	6	12	6	109	71	95	8.4	107
15	7	12	7	102	70	94		
16	5	12	7	107	72	96	7.8	100

Material and Methods

The study was made in five healthy subjects, three males aged 20—46 years and two females 42 and 44 years of age. Three were healthy volunteers and the other two had undergone cardiac catheterization for the suspicion of heart failure and were found to be normal.

Two substance P preparations have been used, both prepared by the same method (PERNOW 1953) and assayed against the same standard. The preparation used in the first two subjects and made by us, had an activity of 550 units per mg. The second preparation, 213 units per mg, was prepared and kindly put at our disposal by Hoffman-La Roche A. G., Basel, Switzerland. Substance P activity was expressed in units according to EULER (1942). Identical subjective and objective reactions were obtained with the two preparations.

Cardiac output was determined by the direct Fick method. Expired air was collected in Douglas bags. Arterial and mixed venous oxygen content and capacity were analyzed spectrophotometrically. Analysis of variance gave a standard error of 0.1 ml O_2 per 100 ml blood. The standard error of the cardiac output determination as performed in this laboratory is 8 per cent (HOLMGREN and PERNOW 1959).

Procedure

The subjects were supine and studied in the morning in as basal conditions as possible. A double lumen catheter was inserted into the right heart from a cubital vein. A polyethylene catheter was inserted percutaneously in the brachial artery. Pressure recordings from the right ventricle, pulmonary artery, pulmonary arterial wedged position (PCV) and brachial artery as well as duplicate determinations of the cardiac output were made at rest.

The substance P preparations were dissolved in saline and given intravenously in amounts of 0.2—1.2 units/kg/min. The infusions lasted 10 min during which time cardiac output was determined and pressures recorded.

Results

General symptoms

Immediately after starting the infusion the subjects felt warmth of their head and temporal pulsations were felt. Simultaneously a bright red flushing was observed in the face and the neck. These symptoms reached a maximum 1—2 min after starting infusion, lasted another 2 min and then slowly disappeared in spite of a constant infusion rate. In a few cases the symptoms returned but to a milder degree when the infusion rate was doubled. No further reactions were observed.

Pulse rate

The pulse rate immediately increased during infusion of substance P with doses of 0.3 units/kg/min or higher. Generally there was a greater increase of the pulse frequency with increasing doses. Tachycardia was maximal 2 min after the start of the infusion. In the next 4 min the pulse rate was unchanged. Normal pulse rates resumed 1—2 min after stopping the infusion (Table I).

Blood pressure

Right ventricular and pulmonary artery pressures did not change during substance P infusion. The brachial artery pressure, however, dropped immediately after the start of infusion. Minimal values were recorded within 2 min, after which the arterial pressure increased (Case 4 and 5). No further decrease was observed with increased infusion rate. When the infusion was repeated 15—30 min later, the same fall in arterial pressure was, however, obtained (Case 3 in Table I). The resting level again resumed within one minute after the end of the infusion (Case 3, Table I).

Oxygen uptake

No change was observed in pulmonary oxygen uptake during infusion of substance P.

A. V. — O_2 difference

During substance P infusion the total arterio-venous oxygen difference, as calculated from brachial and pulmonary artery blood, decreased slightly. At an infusion rate of 0.6—0.9 units/kg/min this decrease was 6—9 ml per litre.

Cardiac output

At an infusion rate of 0.4 units/kg/min and more the cardiac output increased slightly in all cases. The stroke volume increased only in one case, while no significant changes were observed in the others (Table I).

Discussion

The main circulatory effect of substance P seems to be peripheral vasodilatation with flushing and fall of arterial blood pressure. A direct effect on the vessels of substance P is in agreement with the observations by HOLTON and HOLTON (1952). The tachycardia is probably a result of hypotension and does not necessarily involve a cardiotropic action.

Stroke volume was not significantly changed during substance P infusion and the increased cardiac output was due to the tachycardia. The peripheral vasodilatation probably effects a displacement of blood from the lungs to the systemic circulation but not enough to impair the diastolic filling of the left ventricle.

It has previously been observed (LILJEDAHL *et al.* 1958), that the flushing, tachycardia and fall in blood pressure following substance P infusion were transient and disappeared even during continued infusion. These observations have been confirmed in this study. Both the subjective feelings of the patients and the hemodynamic changes were maximal during the first minutes of continuous infusion. At higher infusion rates no further increase in pulse

rate nor any further fall in blood pressure was recorded. It is interesting to note that such tachyphylaxis was not obtained with the action of substance P on the smooth muscle in the digestive tract since a continuous increase in intestinal peristalsis was observed at raised infusion rate (Liljedahl *et al.* 1958). The tachyphylaxis was, however, transient and infusions repeated after 15—30 min gave the same hemodynamic effects.

The two substance P preparations used in this study are not chemically pure, but from preceding pharmacological studies the presence of other biologically active principles has been excluded. Furthermore identical hemodynamic effects were obtained in this study with the two preparations. In an earlier report, it was pointed out that no intestinal or vascular effects were observed after infusion of inactivated preparations (Liljedahl *et al.* 1958).

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The Specificity of Afferent Cutaneous C Fibres in Mammals

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Abstract

IRIUCHIJIMA, J. and Y. ZOTTERMAN. *The specificity of afferent cutaneous C fibres in mammals.* Acta physiol. scand. 1960. *49.* 267—278. — By dissecting very fine strands of cutaneous nerves in rats, dogs and cats it has been possible to record spikes from single afferent C fibres when applying different stimuli to the skin. Confirming earlier investigators, records have been obtained of the activity of C fibres which responded specifically to tactile stimulation of the skin and from C fibres which responded rather unspecifically to strong thermal changes in the skin in both directions ($\pm$ 10° C) as well as to mechanical stimulation of the skin.

In addition to these fibres a new type of C fibres were found which responded specifically to warming or to cooling the skin to less than 1° C. These specific "warm" C fibres and specific "cold" C fibres showed in general a behaviour similar to that of δ thermal fibres previously described. They did not respond to touching their receptive field. The skin of the mammals investigated thus possess specific "warm" C fibres as well as specific "cold" C fibres which are reciprocally brought in and out of action when the skin is subjected to even slight temperature changes.

During the early electrophysiological studies of mammalian cutaneous fibres it became quite obvious to one of us (ZOTTERMAN 1933) that pain must be mediated not only by A fibres (principally δ fibres) but also by C fibres, and from reaction time measurement on human subjects it was concluded that C fibres were associated with the "second" pain sensation. Previously ADRIAN (1931) applying noxious stimuli to the skin of the frog had recorded slow axon

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potentials which most likely were produced by afferent C fibres. In 1935 CLARK, HUGHES and GASSER could prove that a stimulus exciting C fibres as well as A fibres produces larger nociceptive reflexes than one exciting only A fibres. Also it was shown that the A fibres are not necessary for the production of the reflexes, as the latter can be evoked when all the A fibres have been blocked by a pneumatic cuff surrounding the nerve. Some years later (ZOTTERMANN 1939) it was demonstrated that all kind of mechanical stimulation of the skin such as stroking, needle prick, as well as burning stimuli produced not only spikes from A fibres but also a considerable number of C spikes, which could be seen particularly easily in the after-discharge to these stimuli as well as when applying a burning stimulus avoiding mechanical stimulation. These studies thus led to the conclusion that C fibres in mammalian cutaneous nerves respond to various modes of stimulation.

Lately DOUGLAS and RITCHIE (1957) developed their method of studying C fibre activity by recording the reducing effect of different modes of skin stimulation on the antidromic C elevation produced by an electric shock stimulus. In a recent paper (DOUGLAS and RITCHIE 1959) these authors have demonstrated that a large mass of C fibres can respond to quite innocuous mechanical as well as to thermal stimuli and that C fibres obviously serve different sensory modalities.

A substantial step further was provided by IGGO (1959 a, b) who was the first to secure single C fibre preparations from mammalian cutaneous nerves. He was able to prove that a great number of C fibres obviously responded to mechanical stimuli (touch and pressure) and that these fibres to a great extent seemed to be specific to mechanical stimuli *i. e.* they did not respond to heating or cooling, while he found other C fibres responding to skin thermal stimuli although only to rather great changes ($\pm$ 10° C) (IGGO 1959 b). These C fibres show thus a quite different behaviour to that of the thermal δ fibres of the tongue described by ZOTTERMAN (1936) and by HENSEL and ZOTTERMAN (1951) and by DODT and ZOTTERMAN (1952) (see Handbook of Physiol. Section 1. Neurophysiol. 1959. p. 431). In fact IGGO (1959b) suggests that these C fibres described by him may mediate pain.

The present investigation was carried out in order to study more in detail the specificity of the cutaneous C fibres particularly whether there existed any specific thermal fibres *i. e.* C fibres, which responded to thermal changes as specifically as the δ thermal fibres previously described.

Materials and Methods

A total of 41 white rats, 7 dogs and 3 cats were used in this study. The rats and the dogs were anaesthetised by intraperitoneal injection of 6 per cent Mebumalnatrium solution containing 1.8 g Pentobarbitone, 4 g Pentobarbitone sodium and 25 g Urethane in 100 ml. The injected dose was 0.4 ml of this solution per kg body weight. The cats were

Table I

Fibre no.	Conduction velocity m per sec	Touch	Cooling	Warming
1	..	—	+ (35–34)	—
2	..	—	+ (33–30)	..
3	..	—	+ (33–32)	—
4	..	—	+ (36–35)	..
5	..	—	+ (33–32)	—
6	..	—	+ (35–31)	..
7	0.9	—	+ (35–33)	..
8	0.8	—	+ (34–30)	..
9	..	—	+ (37–30)	..
10	1.0	—	+	..
11	0.8	—	+ (36–28)	..
12	0.6	+	..	+
13	0.6	—	..	+
14	..	—	..	+ (35–37)
15	..	—	..	+
16	1.1	+	..	+ (37–48)
17	0.5	+	+ (36–8)	+ (36–55)
18	0.9	+	..	+ (36–52)
19	0.4	+	—	—
20	1.0	+	..	+ (36–50)
21	1.0	+	..	+ (36–56)
22	0.9	+	+ (35–7)	..
23	..	+	+ (38–8)	..
24	1.0	+	+ (37–15)	..
25	1.5	+	..	+
26	1.5	+	..	+ (38–40)
27	0.7	+	..	+ (38–41)
28	..	—	+ (39–36)	
29	0.8	+	+ (34–8)	+ (34–58)
30	1.0	+	..	+ (36–58)

anaesthetised by intramuscular injection of a chloralose-urethane solution (0.05 g chloralose and 0.25 g urethane in 7 ml of Ringer's solution per kg body weight).

All experiments on the rats were carried out on the saphenous nerve while in the dogs and the cats we also worked on the infraorbital nerves supplying the nose of the animal.

The nerve was placed on a small platform with a black surface offering a good contrast to the nerve strands. The wound was filled with paraffin oil at 37° C and the nerve strands were lifted on to a single or a double platinum electrode. When spikes were obtained from single C fibres to physiological stimulation of the skin the conduction rate was determined by applying an electric stimulus to their receptive fields. In most experiments the identity of the electrically evoked C spike could be ascertained by the identical height and configuration of the spikes produced by the natural stimulation of the skin using the same speed of recording as will be seen from Fig. 1.

Under paraffin oil, nerve preparations containing only one or a few active C fibres

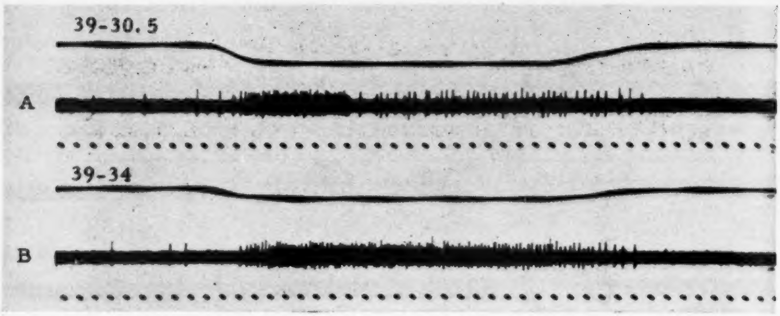


Fig. 4. Showing that the first high frequency discharge and the following slow one, observed on extreme cooling, are of one and the same fibre. Time mark 10/sec.

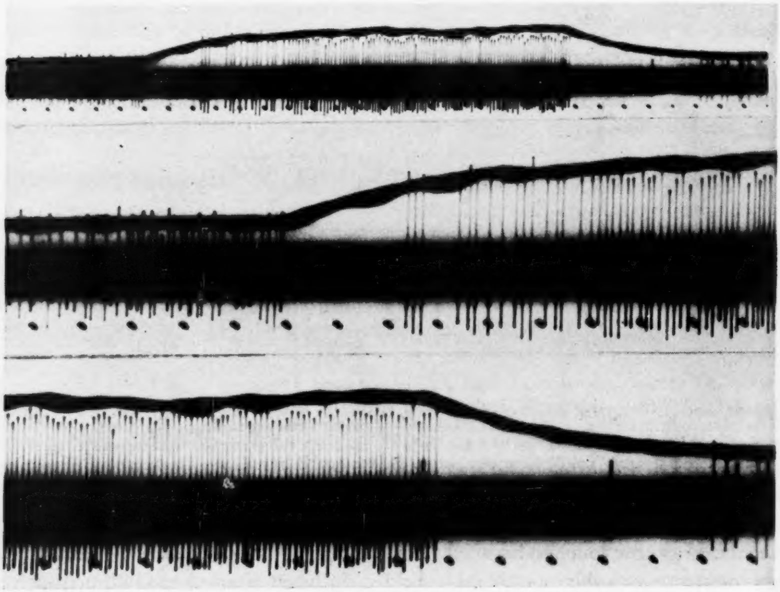


Fig. 5. Record from a fine strand of the infraorbital nerve of the cat showing the behaviour of a few "cold" fibres and a single "warm" fibre when the skin of the nose is exposed to infrared rays slowly raising the temperature from 33° to 39° C. Time marks 1 sec.

followed by a short silent period. At moderate rates of cooling (Fig. 4 B), however, this fibre does not discharge at such a high rate as to produce any peripheral inhibition.

Similar records from "cold" fibres have been obtained from the saphenous

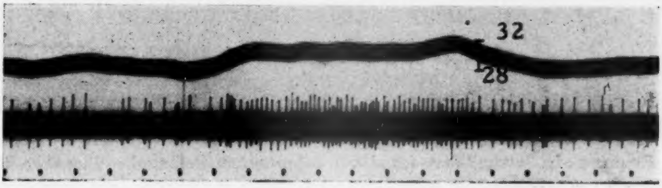


Fig. 6. Response of a "warm" fibre in dog's infraorbital nerve to radiant heat. Time in 1 sec.

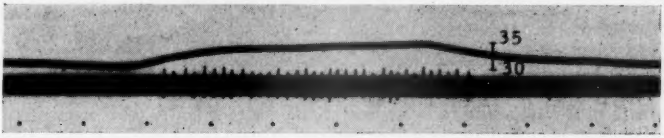
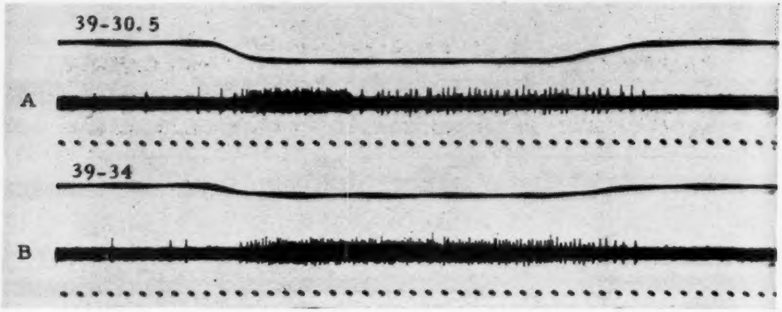


Fig. 7. Response of a "warm" fibre in dog's saphenous nerve to radiant heat applied to the skin. Cond. vel. 0.4 m/sec. Time in 1 sec.

nerve as well as from the infraorbital nerve of the dog and the cat. Fig. 5 shows the response from a fine strand of the infraorbital nerve of the cat supplying the skin of the upper lip and the tip of the nose. In this record we see the signalling of a few "cold" fibres at a skin temperature of 33° C. This discharge of "cold" fibres was almost instantaneously stopped by exposing the skin to infrared radiation. When the skin temperature had risen to about 36° C another fibre producing spikes of about double the spike height started to discharge at an increasing rate. When now the lamp was switched off this "warm" fibre response ceased immediately, to be followed after a certain silent period by a discharge from the "cold" fibres as the temperature fell. Unfortunately the rate of conduction of these fibres was not measured but the records prove, however, that there were two kinds of specific thermal fibres responding reciprocally to warming and cooling the skin. None of these fibres responded to touch.

In the dog we found "warm" C fibres in the infraorbital nerve which, unlike the above described "warm" fibre of the cat, displayed a steady discharge already at the relatively low temperature level of the skin of 28° C as will be seen in Fig. 6. A rise from 28° to 32° C gave a very substantial rise in the frequency of spike discharge of this fibre. A "warm" C fibre of a conduction rate of 0.4 m per sec was also found in the dog's saphenous nerve. This fibre did not display any steady discharge at a skin temperature of 30° C but responded very nicely to a rise of a few degrees Celsius (Fig. 7).

Besides these "warm" C fibres which behaved similarly to the "warm" δ fibres of the tongue, described by Zotterman (1936) and Dodt and Zotterman (1952) we encountered another type of fibres which responded only at higher temperature levels. Typical examples of such fibres are given in Figs 8 and 9. In both these records from saphenous nerves of rats we notice a sudden burst



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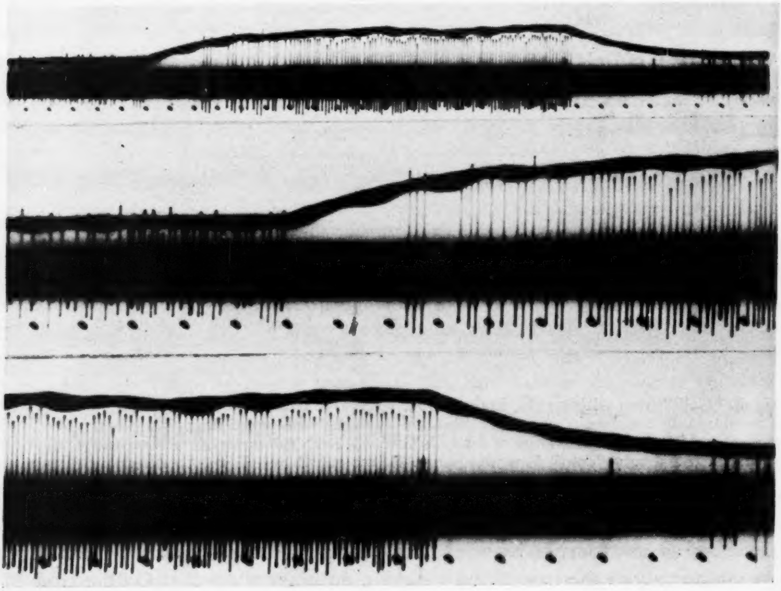

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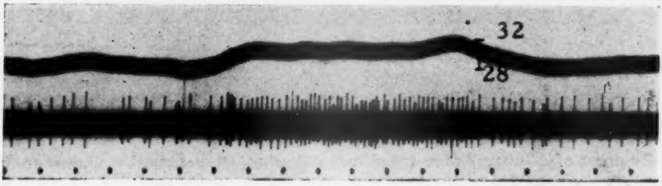
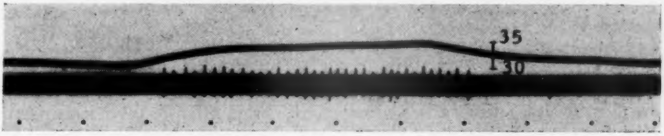


Fig. 6. Response of a "warm" fibre in dog's infraorbital nerve to radiant heat. Time in 1 sec.



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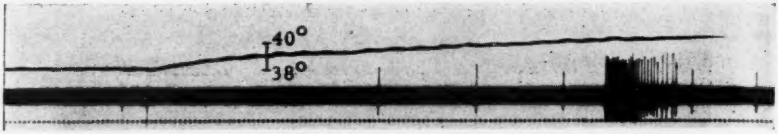

Fig. 8. Response of a "warm" fibre (cond. vel. 0.6 m/sec) and of another fibre to radiant heat. Temperature was recorded with a thermojunction placed on the skin. Time mark 100 msec.

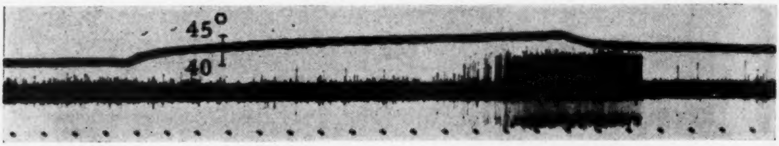

Fig. 9. High frequency discharge of a fibre responding to radiant heat. Time mark 1 sec.

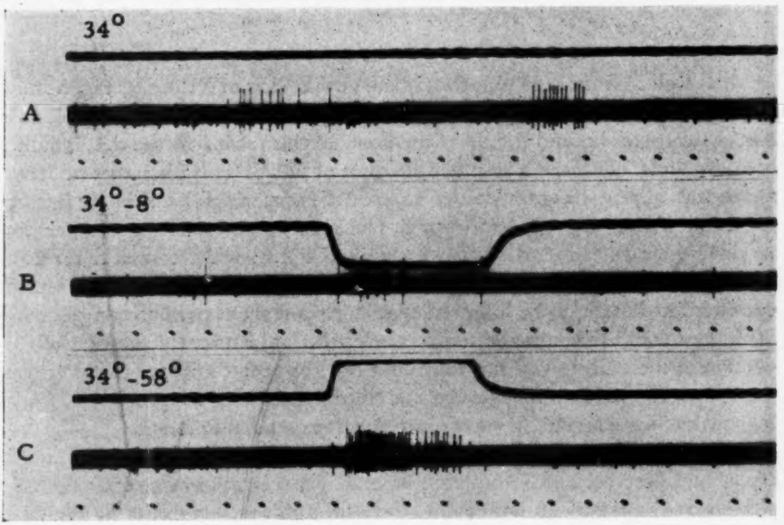

Fig. 10. Response of "pain" (?) fibre conducting at 0.8 m/sec to (A) pressure on thermode (34° C), (B) extreme cold (34°—8° C) and (C) extreme warmth (34°—58° C). Time mark 1 sec.

of spikes of high frequency when the temperature reaches a level of 41° C respectively 43° C. These responses have the character of an injury discharge and such bursts were seen in "cold" fibres as well in response to very rapid cooling of the skin. It is difficult to tell whether these fibres should be classified as specific "warm" fibres as they only respond at fairly high skin temperatures but they differ from the more unspecific C fibres to be described below in that they did not react to touch or rapid cooling.

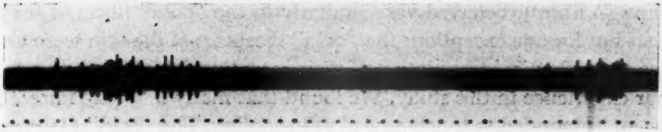

Fig. 11. Response of a C fibre to light touch with the tip of a brush. Cond. vel. c. 0.9 m/sec. Time 100 msec.

Fibres responding to extreme temperatures. Such fibres were found fairly often but we did not record the response from more than a few of them. The behaviour of such a fibre is seen in Fig. 10. This fibre conducting at a rate of 0.8 m per sec responded to heating the skin above 50° C. It also signalled on cooling the skin to low temperatures. All fibres of this type of behaviour were also stimulated by mechanical stimuli applied to skin such as touch, pressure, pinching and pin prick. These fibres are obviously of the same type as those recently described by IGGO (1959 b). Such fibres would thus not serve any fine thermal discrimination but might very well induce nociceptive reactions, as this author also suggests.

Fibres responding specifically to touch. A great number of such C fibres were encountered in all the nerves investigated. Such a fibre from the rat's saphenous nerve is seen in Fig. 11. These fibres do not respond to moderate thermal changes in the skin. Such fibres have been described previously by one of us (ZOTTERMAN 1939) and more recently by DOUGLAS and RITCHIE (1958) and by IGGO (1959 b). They seem to be as specific for touch as the A touch fibres. Whether a selective stimulation of such C fibres would produce a sensation of touch is a question which will be discussed below. We are rather inclined to believe that they might mediate tickling or itch (cf ZOTTERMAN 1939).

Discussion

The specific thermal C fibres studied in this investigation are apparently different from the C fibres described recently by IGGO (1959). His thermal C fibres responded only to quite strong cooling or heating of the skin ($\pm$ 10° C). Such fibres were also observed by us, but in addition to these we encountered a number of C fibres which were sensitive to as slight a cooling of the skin as of less than 1° C. These specific "cold" C fibres displayed a steady discharge within different temperature ranges. Thus some of these "cold" C fibres showed a steady discharge even at as high a temperature as 38° C, while the upper level for the steady discharge of other "cold" C fibres was only 28° C. Therefore even in this aspect these "cold" C fibres behaved very similarly to those described by HENSEL and ZOTTERMAN (1951).

Recently BOMAN (1958) in Hensel's laboratory made an extensive study of thermal receptors of the skin of the face of rats, cats and dogs recording from fine strands of the infraorbital nerve. He noticed that the fibres which responded

to cooling (δ fibres), behaved very similarly to the "cold" fibres of the tongue of the cat but for one exception: the "cold" receptors of the skin were generally sensitive to mechanical stimulation of their receptive field. This is in agreement with our experience in this study. We found that many δ "cold" fibres, but not all of them, responded to touch as well as to cooling. The "cold" C fibres on the contrary, were insensitive to touch as well as to pressure within reasonable limits of stimulus strength. Therefore the skin of the animals studied apparently possesses myelinated as well as non-myelinated afferent fibres, the endings of which respond specifically to cooling of the skin.

Specific fibres responding to warming the tongae a few degrees have only been recorded by ZOTTERMAN (1936) from the lingual nerve and by DODT and ZOTTERMAN (1952) from the chorda tympani of the cat. Apparently these fibres were δ fibres. In this investigation we have recorded the integrated response from the whole chorda tympani of the dog to warming the tongue stepwise 2° to 3° C from 30° to 38° C and we obtained a positive response even to warming the tongue from 30° to 33° C. Thus we are not in doubt as to the existence of specific δ "warm" fibres in the tongue. BOMAN (1958) failed to observe any similar "warm" fibres from cutaneous mammalian nerves. If the relative distribution of "warm" and "cold" fibres were similar to the density of the warm and cold spots in the human skin (c. 1 : 10 in the upper lip) then the chance of obtaining an isolated warm fibre or a fine nerve strand containing a "warm" fibre apparently would be rather small. That we have found comparatively few "warm" fibres is thus only what might be expected. One of these "warm" fibres from the infraorbital nerve of the cat (Fig. 5) is most likely a δ fibre. The other "warm" fibres which we were able to study in this research, altogether only 3 fibres in the rat and 5 fibres in the dog, were C fibres with conduction velocities varying from 1.5 to 0.4 m per sec.

These "warm" C fibres react quite promptly to slight temperature rises of less than one degree Celsius within a temperature range of 28° to 42° C. Even a very slight cooling stops their discharge and they are not sensitive to touch nor to pressure within reasonable limits of stimulus strength. They display thus all the properties which one should require from a "warm" receptor. Taken together with the fact that C "cold" fibres generally do not respond to mechanical stimulation of the skin we find it fairly obvious that the warm-blooded animals examined in this investigation possess a dual mechanism of thermal receptors which should be sensitive enough for developing a thermal discrimination like that of human beings. We do not know as yet whether among the thermal fibres the C fibres fulfil a specific function of a sensory or a thermoregulatory character different from that of the medullated thermal fibres previously studied in more detail. For that purpose we ought to know more about the central connections of the C fibres, a field of our knowledge which still is more or less a blank. Likewise for obvious reasons we lack quantitative data about the distribution of specific "warm" and "cold" fibres in the skin of

animals, enabling us to say anything definitely in regard to the relative importance of δ and C fibres respectively in thermal discrimination or in the regulation of the body temperature. But the fact that afferent C fibres are very numerous in cutaneous nerves makes it highly probable that these fibres play a rather important rôle and we would like to venture that that may apply particularly to the "warm" C fibres.

The question about what particular purpose the C fibres responding to light touch would serve, has recently been the subject of a detailed discussion (see "Pain and Itch", Ciba Found. Study Grp. no. 1. 1959). These fibres end apparently very superficially, shooting their endings out between the epithelial cell of the epidermis (WEDDELL *et al.* 1959). The topography of these endings would thus suggest that they should be sensitive to very minute deformations of the surface such as by light touch. Very light touch, particularly when it is repeated, gives rise to superficial tickling or itching sensations and it seems very likely that such fibres really mediate tickling or itching when they are selectively stimulated.

LELE and WEDDELL (1959) still maintain their idea that there are no specific sensory fibres in the hairy skin. They base this statement on the fact that their histological examination of the hairy skin does not reveal any encapsulated endings but only naked nerve endings or a network of nerve fibres. They claim that "there is no evidence to support the hypothesis that the free nerve endings in human hairy skin are physiologically specialized; indeed a great deal of evidence has accumulated to support the hypothesis that the free nerve endings in skin are physiologically non-specialized and discharge nerve impulses in different spatiotemporal patterns on stimulation of the skin by different types of stimuli".

The fact that C fibres, which as far as we are aware, do not possess any encapsulated endings are found to be quite specific to touch, warmth and cold tells us that the specificity can hardly be coherent to the capsulation. Several years ago one of us (ZOTTERMAN 1953) drew attention to the fact that the tongue of the frog possesses quite specific fibres for salt and water as well as for mechanical stimuli (touch and pressure) in spite of the fact that it contains no encapsulated nerve endings. From this it was suggested that the capsules may play another rôle *i. e.* to protect the naked nerve endings. Recently LOEWENSTEIN and RATHKAMP (1958) managed to peel off all the adventitious structure of Pacinian corpuscles leaving only the inner core with its enclosed nerve ending and axon intact. They could prove that this procedure did not change the responsiveness of the endorgan. Thus it seems quite obvious that the structures responsible for the receptor specificity *i. e.* its transducing activity is not coherent to the capsule but as LOEWENSTEIN (1959) states is scattered all over the non-myelinated ending. The specificity of the afferent nerve endings in the hairy skin will thus very likely be revealed only by differences in their molecular structure.

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The Effect of Body Position on the Circulation at Rest and During Exercise, with Special Reference to the Influence on the Stroke Volume

By

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Abstract

Bevegård, S., A. Holmgren and B. Jonsson. *The effect of body position on the circulation at rest and during exercise, with special reference to the influence on the stroke volume.* Acta physiol. scand. 1960. *49*. 279—298. — In 10 healthy, adult, male subjects the hemodynamics have been studied by the aid of heart catheterization at rest and during work both in supine and sitting positions. The cardiac output was on the average 2.2 l/min less in the sitting than in the supine position, both at rest and during exercise. In the supine position, the stroke volume was constant at rest and during exercise. In the sitting position, the stroke volume at rest was 40 per cent smaller than in the supine. It increased considerably with mild work, but not significantly with an even heavier work load. It never reached the values obtained in the supine position, even during heavy work. The arterio-venous oxygen difference was larger in the sitting than in the supine position, both at rest and during work. During heavy work, the oxygen transport per heart beat was the same in the supine and the sitting positions, and the physical working capacity (work performed per heart beat) was also of the same order.

In a recent study Holmgren, Jonsson and Sjöstrand (1960) found that the stroke volume of the heart in the supine position remained rather constant during work and was equal to that at rest. In the earlier literature on this subject, however, contradictory results have been reported (for references see Holmgren *et al.* 1960). This could be explained to some extent by the fact that some studies were made in the supine and some in the upright position.

Table I. Some anthropometric data of 10 healthy male subjects

Case no.	Age, years	Height, cm	Weight, kg	B.S.A., m^2	Heart volume, ml	Total hemoglobin gms	Total hemoglobin gms/kg	Blood volume, l.	Work intensity, kpm/min, at pulse rate 170 beats/min: Sitting, Before cath.	Sitting, During cath.	Supine, Before cath.	Supine, During cath.	Pulse rate (standing) beats/min
1	23	185	79.0	2.05	885	940	11.9	6.3	1,000	—	1,050	970	92
2	23	182	79.5	2.01	945	870	10.9	6.3	1,030	[1] 980	1,110	—	96
3	25	180	71.5	1.92	1,020	745	10.4	5.6	1,260	[2] 1,530	1,330	[1] 1,330	80
4	27	181	63.3	1.81	720	460	7.3	4.2	570	600	660	[1] 560	104
5	25	182	78.0	2.01	850	805	10.3	6.0	1,160	1,200	[1] 910	[1] 1,090	77
6	24	171	63.6	1.76	880	670	10.5	5.3	920	[1] 830	[1] 840	[1] 760	101
7	28	181	90.5	2.12	975	795	8.8	6.2	950	1,190	1,050	[1] 1,080	71
8	21	183	65.0	1.87	865	805	12.4	6.3	1,130	[1] 1,020	1,080	1,160	78
9	20	173	66.5	1.80	755	630	9.5	4.8	[1] 1,040	[1] 950	[1] 890	[1] 920	100
10	41	180	76.0	1.97	970	795	10.5	5.9	1,140	[1] 1,080	[1] 1,080	1,080	86

[1] Not in "steady state".
[2] Extrapolated from pulse rate 130/min.

Holmgren and Ovenfors (1960) found that if the subject was in the supine position, the heart volume was the same at rest and during work of increasing intensity. In the sitting position, the heart volume at rest was smaller than in the supine. During mild exercise, however, it increased to about the same size as in the supine position and remained constant with increasing work intensities.

In the present report, the behavior of the stroke volume at rest and during exercise has been studied on the same subject in the sitting and the supine position, using the direct Fick method.

Material

Ten healthy male subjects were chosen for study. They were all physically active, but did not take part in athletics. Except for case no. 10, all were blood donors registered at the hospital blood bank. Values for age, body weight, height and other anthropometric data are given in Table I. Prior to the investigation all underwent a clinical examination including clinical history, auscultation of heart and lungs, X-ray of the chest, ECG at rest and during work, and routine blood and urine analysis. They showed no signs of disease.

Methods

Before the procedure started, the subjects were informed about the methods and the purpose of the investigation. A few days before heart catheterization the physical working capacity was determined, one day in the sitting position and next day in the

supine position. Heart volume and total amount of hemoglobin (THb) was also determined before heart catheterization.

Electrocardiogram: The technique is described in detail elsewhere (HOLMGREN *et al.* 1959).

Orthostatic test. Pulse frequency and ECG were recorded after standing for 8 min.

Physical working capacity (PWC). This was determined in sitting as well as in supine position according to SJÖSTRAND (1947) and WAHLUND (1948) on an electrically braked bicycle ergometer (HOLMGREN and MATTSSON 1954). The PWC_{170} is defined as the working intensity in kpm per minute which the subject could perform at a pulse rate of 170 beats per minute. The work load was increased stepwise every 6 min until a pulse rate of about 170 was obtained. Using the approximately linear relationship between pulse rate and work load, the value of the PWC_{170} is obtained by inter- or extrapolation. In calculating the PWC_{170}, no correction was made for a "non-steady state" with regard to the pulse rate at the highest work load as in earlier clinical studies (HOLMGREN *et al.* 1958). A relative steady state was defined as being present if the pulse rate did not increase more than 10 beats from the second to the sixth minute of work, or if it exceeded this value did not increase more than 3 beats between the fourth and sixth minute.

Total amount of hemoglobin (THb). The THb was determined with the alveolar CO-method (SJÖSTRAND 1948). Duplicate determinations were made at an interval of one day. During the period of this investigation, the error of a single determination varied between 3.5 and 4 per cent.

Blood volume. The total blood volume was calculated from the THb and hemoglobin concentration (Hb) of finger blood, disregarding the slight error due to the assumption of a constant hemoglobin concentration in the whole blood volume.

Heart volume. Heart volume was determined in the prone position according to the method described by KJELLBERG, RUDHE and SJÖSTRAND (1949) and LARSSON and KJELLBERG (1948). The standard error of a single determination amounts to 4 per cent.

Right heart catheterization. This was carried out in the usual manner. A polyethylene catheter was introduced into the brachial artery by a percutaneous technique (SELDINGER 1953). For heart catheterization a double-lumen catheter was used in all cases but one. *Blood pressures* were recorded by a Swema-Elema strain-gauge mechano-electrical transducer and recorded on an Elema "Klinik" ECG-apparatus (HOLMGREN 1956). Mean pressures were obtained by means of electric integration. The reference point for zero pressure in the supine position was taken as 5 cm below the insertion of the fourth rib at the sternum. In the sitting and standing positions, the insertion of the fourth rib at the sternum was regarded as the reference point for zero pressure.

During exercise in the sitting position, the subject was leaning against a support, to keep the reference level as constant as possible. Cardiac output was measured according to the direct Fick method. Oxygen uptake at rest and during work was determined by the Douglas bag technique. Expired air at rest was collected for 10 min and during work for 3 min, between the third and sixth minute of work on each load, after a relative steady state had been obtained. Simultaneous blood samples were drawn slowly from the pulmonary and brachial arteries during the collection of the expired air. The expired gas volume was measured with a gasometer. The gas samples were analysed according to HALDANE and PRIESTLY (1935).

Blood gas analysis. O_2-saturation and hemoglobin concentration were measured spectrophotometrically (HOLMGREN and PERNOW 1959). The oxygen content was determined from the oxygen saturation and the oxygen capacity of the blood sample, adding the amount of physically dissolved oxygen, estimated according to PETERS and VAN SLYKE (1932). The oxygen capacity was calculated from the hemoglobin concentration using the factor 1.34 for the oxygen binding capacity of hemoglobin. With the described

technique, HOLMGREN and PERNOW (1959, 1960) found the error of a single determination of cardiac output to be 8.2 per cent at rest and 5.2 per cent during work. For stroke volume, these values were 8.6 and 6.8 per cent respectively. The oxygen capacity was determined in all blood samples both at rest and during work.

Mechanical efficiency (M). This was calculated according to the formula:

$$M = \frac{\text{work performed} \times 100}{(\text{total energy exchange}) - (\text{basal energy exchange})}$$

The total energy exchange was calculated from the O_2-uptake, assuming the caloric value of O_2 to be 4.9 kcal/l.

Procedure

The volunteers had a light meal in the morning, but received no sedative or quinidine. They arrived at the hospital at 8 a. m. and rested in the supine position one hour before the examination started. When the catheters had been introduced, carciac output and blood pressures were measured at rest in the supine position. The subject was then allowed to sit up on the catheterization table for about half a minute in order to get accustomed to altered body position. After that, he walked a few steps and mounted the bicycle ergometer. Blood pressures and cardiac output were determined after approximately 6 min rest in the sitting position. As soon as these measurements were completed the work test began. Cases no. 3—10 performed two work loads and cases no. 1 and 2 three loads with increasing intensity. The work loads were chosen with the guidance of the earlier determined physical working capacity at pulse rate 170 beats per minute (PWC_{170}) so that the final pulse rate on the highest work load was about 150 beats per minute. On each load, pressures and flow were determined with the described technique. When the investigation in the sitting position had been completed the subject was again brought to the catheterization table. After he had rested for 30 min in the supine position, flow and pressures were again measured. This was followed by determinations during work in the supine position on the same work loads as in the sitting position. In case no. 1 and 2, measurements were made in the standing position instead of in the sitting position. Case no. 1 was examined during work only in the supine position and case no. 2 only in the sitting position. One of the subjects (case no. 9) almost fainted while sitting on the bicycle. The exercise was then started immediately and the symptoms rapidly disappeared. In this case, there was no time for determination of cardiac output at rest in the sitting position.

Results

The *ECG* at rest, in standing position, and during and after exercise was normal in all subjects. Only two subjects (case no. 6 and 9) showed a higher than normal pulse response to the *orthostatic test.* The average pulse rate in the standing position was 88.5 beats per minute (range 71—104).

The total amount of hemoglobin (THb) was on an average of 751.5 g (range 460—940) corresponding to 10.3 g per kg body weight (range 7.3—12.4), which is slightly lower than that found by HOLMGREN *et al.* (1960) in healthy males.

The mean value for *the hemoglobin concentration* was 13.2 g per 100 ml blood (range 11.0—15.0), which is a somewhat low figure. Four subjects had a

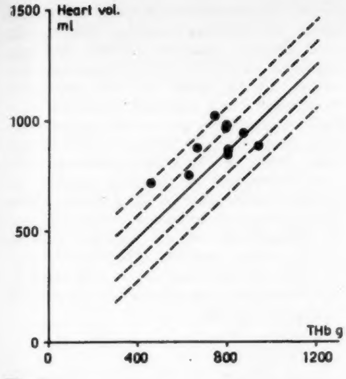

Fig. 1

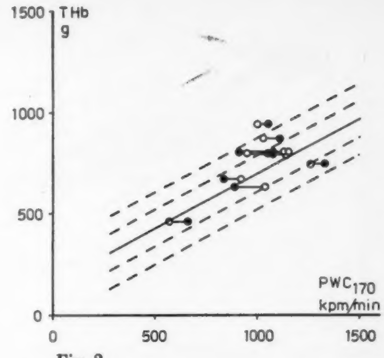

Fig. 2

Fig. 1. Heart volume, ml (ordinate) in relation to total amount of hemoglobin (THb), g (abscissa).

Fig. 2. Total amount of hemoglobin (THb), g (ordinate) in relation to working capacity (PWC_{170}), kpm/min (abscissa). Filled circles denote PWC_{170} in the supine and open circles PWC_{170} in the sitting position.

Fig. 3. Heart volume, ml (ordinate) in relation to working capacity, (PWC_{170}), kpm/min (abscissa). Symbols as in Fig. 2. The straight lines in Fig. 1, 2 and 3 represent the normal regression lines obtained from determinations on 58 healthy subjects. Interrupted lines represent $\pm$ one and two times standard error of estimate (Holmgren *et al.* 1957).

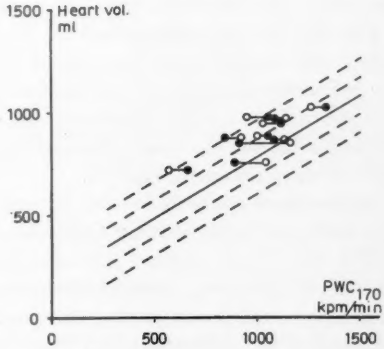

Fig. 3

hemoglobin concentration lower than 13.0 g per 100 ml blood. The probable explanation of the somewhat low figures for THb and hemoglobin concentration is that some of these blood donors had not followed the instruction about iron medication.

The blood volume was 77.9 ml per kg body weight (range 66.4—96.9). This value does not deviate from that found in ordinarily trained young men (Holmgren *et al.* 1960).

The heart volume in the prone position was 886.5 ml (range 720—1,020) and on the average normal when correlated with THb (Fig. 1). Four subjects had a somewhat large heart volume in relation to THb, when compared to a larger group of normal subjects investigated with the same method (Holmgren *et al.* 1957). The deviation from the normal regression line was between one and two times the standard error of estimate. All these cases had a some-

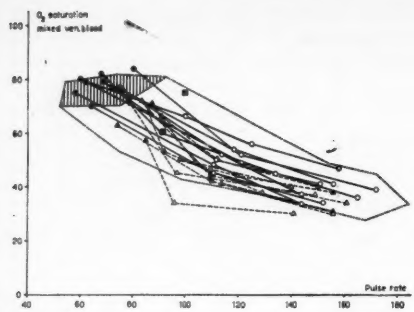

Fig. 4. Oxygen saturation of mixed venous blood, in relation to pulse rate at rest and during exercise. Filled symbols represent values at rest and open symbols values during work. Circles connected by straight lines represent the values for each individual case in the supine position, and triangles connected by dotted lines refer to the sitting position. Two cases examined in standing position at rest are represented by filled squares. The area of normal variation obtained from determinations on 18 healthy subjects in the supine position (Holmgren *et al.* 1960) is surrounded by a dotted line. The hatched area represents the normal variation at rest.

what low hemoglobin concentration. If in these cases the heart volume is correlated to the blood volume, the deviation from the regression line will be less than one standard error of estimate.

The PWC_{170} was determined in the supine and in the sitting positions, before and during right heart catheterization. It is thus possible to evaluate the influence of the heart catheterization on the physical working capacity. It appeared that while exercising during heart catheterization, most of the subjects were not in a steady state with respect to the pulse rate on the heaviest load. This is noted in Table I.

Before heart catheterization, the calculated work load, at a pulse rate of 170 beats per min. in the sitting position, was 1,020 kpm/min (range 570—1,260) and in the supine posture was 1,000 kpm/min (range 660—1,330). This slight difference is insignificant but the individual variations were fairly large (standard error of the difference between means = 112 kpm/min.).

During heart catheterization, the work at a pulse rate of 170 beats per min in the sitting position was 1,028 kpm/min (range 600—1,530) and in the

Table II. The effect of body position on some circulatory functions. Mean values of the material
N = number of cases. S. D. refers to standard error of the difference between means. P =

	PWC_{170} kpm/min		Oxygen uptake ml/min (STPD)			O_2-sat. in mixed ven. blood per cent		
	Before cath.	During cath.	Rest	Work I	Work II	Rest	Work I	Work II
Recumbent	1,000	1,020	289.4	1,160.5	1,960.3	77.3	51.3	39.3
Sitting......	1,020	1,028	347.6	1,124.6	1,958.6	66.9	44.8	35.0
Difference ..	−20	−8	−58.1	35.9	1.6	10.4	6.5	4.3
N..........	10	8	7	8	8	7	8	8
S.D.........	112	114	26.7	79.0	90.8	3.26	2.56	1.98
P..........	>0.6	>0.8	<0.01	>0.2	>0.9	<0.001	<0.001	<0.001

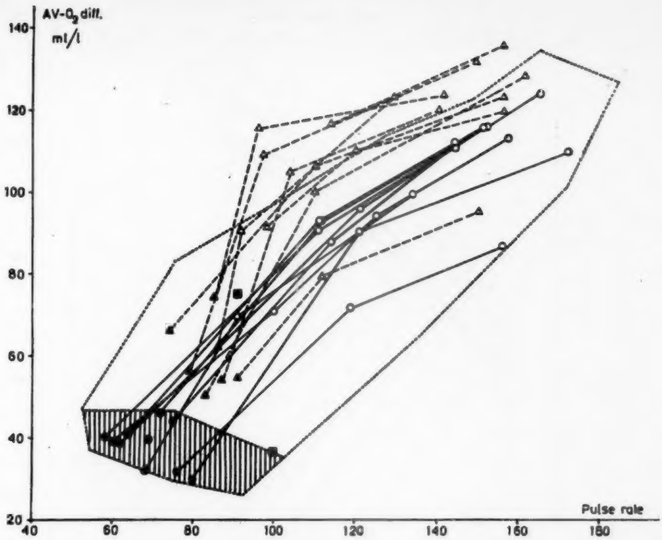

Fig. 5. Arterio-venous oxygen difference, ml/l, in relation to pulse rate, at rest and during exercise. Symbols as in Fig. 4.

supine position was 1,020 kpm/min (range 560—1,330). The difference is not significant. Nor is there any significant difference between these values and those obtained before heart catheterization.

On the average, the PWC_{170} was normal in relation to the THb (Fig. 2). Subjects no. 1 and 2 showed a somewhat low value of the PWC_{170} in relation to the THb. In relation to the heart volume, the PWC_{170} was also a little low for some of the subjects (Fig. 3).

and statistical significance of differences

level of significance

$AV\text{-}O_2$-difference ml/l			Cardiac output l/min			Stroke volume ml		
Rest	Work I	Work II	Rest	Work I	Work II	Rest	Work I	Work II
37.1	86.5	109.2	7.89	13.42	17.93	115.7	120.9	119.3
59.7	104.0	122.5	5.85	10.78	15.94	70.0	104.0	108.4
−22.6	−17.5	−13.2	2.04	2.64	1.99	45.7	16.9	10.9
7	8	8	7	8	8	7	8	8
6.48	6.31	5.67	1.44	0.99	1.44	21.72	14.46	11.22
<0.001	<0.001	<0.001	<0.01	<0.001	<0.01	<0.01	<0.05	<0.05

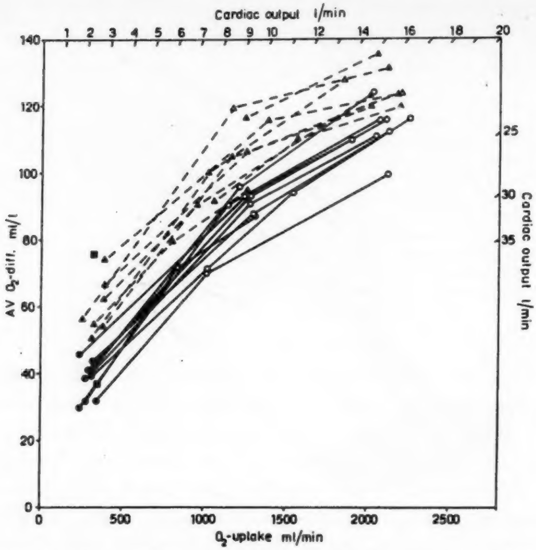

Fig. 6. Arterio-venous oxygen difference, ml/l, in relation to oxygen uptake ml/min at rest and during exercise. The isopleths for cardiac output, l/min, go from origo to the markings on the upper and right scale. Symbols as in Fig. 4.

Data obtained during heart catheterization

The oxygen uptake at rest and in the supine position, was, in the first determination, + 15.9 per cent (range + 5 — + 36) higher than the predicted basal oxygen uptake (Harris and Benedict 1919). The mean value for the determination at rest in the supine position, immediately before the second work test, was + 11.5 per cent (range — 2 — + 30) and at rest, sitting on the bicycle, + 38.3 per cent (range + 3 — + 60).

The pulse rate at rest in the supine position was, during the first determination, 70.0 beats per minute (range 58—78) and during the second 67.8 beats per minute (range 58—80). After 6—7 min at rest, sitting on the bicycle, it amounted to 83.6 beats per minute (range 74—91), corresponding to an increase in pulse rate of 13.6 beats per minute (range 5—27). Subject no. 9, who almost fainted after 6 min in the sitting position, had a systolic pressure in the brachial artery of 55 mm Hg and a pulse rate of 96 beats per minute. The exercise test was then immediately started and the systolic pressure in the brachial artery rapidly rose to about 150 mm Hg and the symptoms disappeared. No determination of the cardiac output at rest could be obtained in this case.

The oxygen uptake during work increased normally with increasing work intensity. There was no significant difference in oxygen uptake between work in the sitting and supine positions. As the work loads were the same, it follows

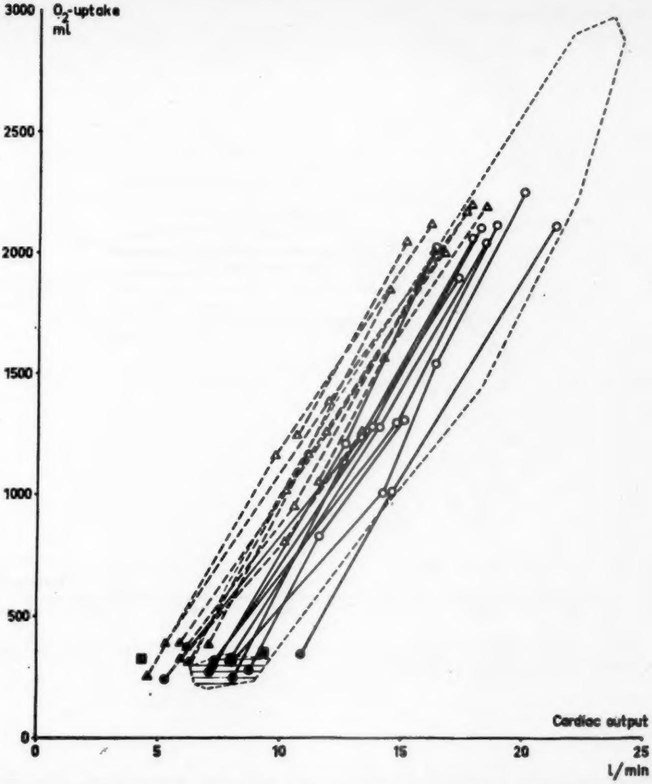

Fig. 7. Oxygen uptake, ml/min, in relation to cardiac output, l/min, at rest and during exercise. Symbols as in Fig. 4.

that the mechanical efficiency was equal. The mechanical efficiency on the highest work load was on the average 23.6 per cent (range 20—25) in the sitting position and 23.5 per cent (range 21—25) in the supine position.

The oxygen saturation of the arterial blood varied within normal limits at rest as well as during work. The increase of the oxygen capacity of the arterial blood during exercise was significant and amounted to 0.89 vol % in sitting and 1.26 vol % in the supine position.

The oxygen saturation of mixed venous blood at rest as well as during work was, in each case, lower in the sitting than in the supine position (Fig. 4). At rest it amounted to 77.3 % (range 70—84) in the supine and 66.9 % (range 57—72) in the sitting posture. This decreased during the highest work intensity to 39.3 % (range 34—45) and 35.0 % (range 30—40) respectively. The

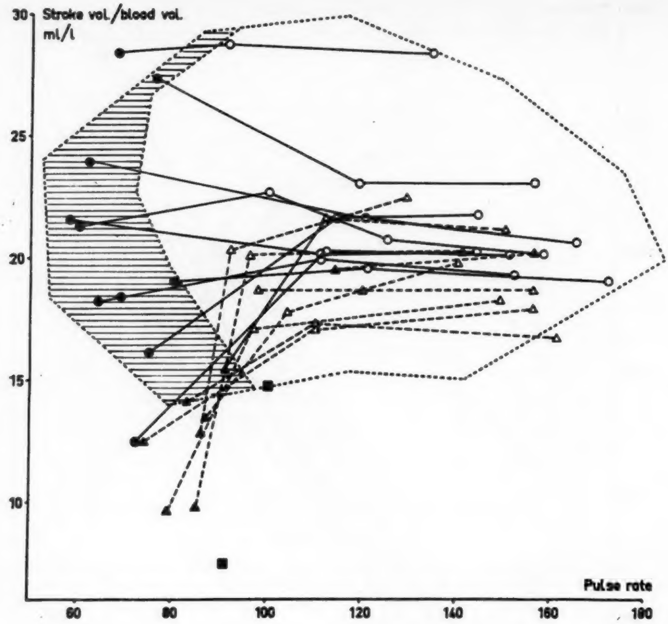

Fig. 8. Stroke volume divided by blood volume, ml/l in relation to pulse rate at rest and during exercise. Symbols as in Fig. 4.

differences between the sitting and supine positions are statistically significant (Table II). During the change from rest to exercise, the saturation of mixed venous blood in relation to work intensity showed a steeper decrease in the sitting position. During continued work with increasing intensity, however, the decreases were approximately parallel in both postures (Fig. 9).

The arterio-venous oxygen difference at rest was 37.1 ml/l (range 31.5—46.2) in the supine and 59.7 ml/l (range 54.1—74.3) in the sitting position. It increased during the highest work load to 109.2 ml/l (range 86.6—123.9) and 122.5 ml/l (range 95.0—135.5) respectively. In each case the arterio-venous oxygen difference at rest, as well as during work, was higher in the sitting than in the supine position (Fig. 5). The differences were statistically significant (Table II).

In the sitting posture, the arterio-venous oxygen difference showed a steeper increase on changing from rest to work than in the supine position. During continued work with increasing loads, the increases were approximately parallel in both postures (Fig. 9).

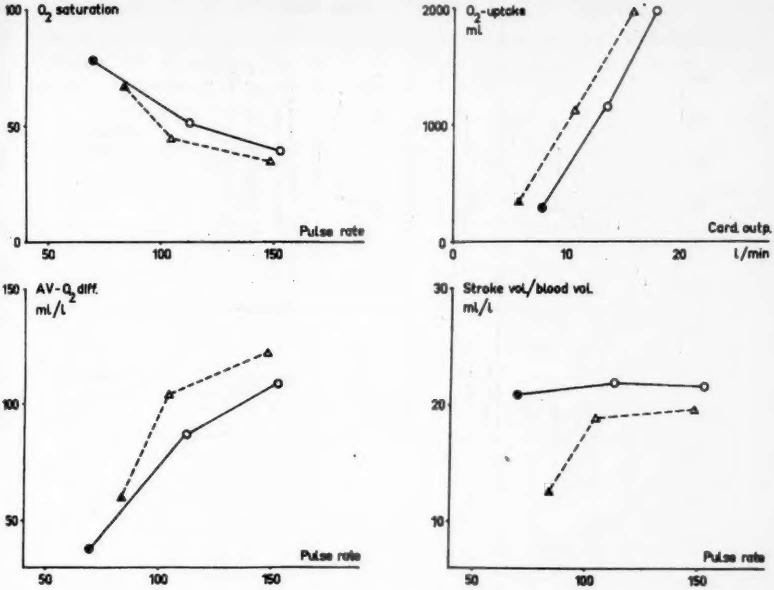

Fig. 9. Mean values for cases no. 3—8 at rest and during exercise. Symbols as in Fig. 4.

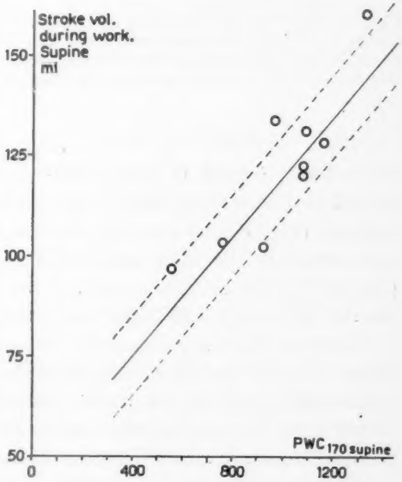

Fig. 10. Stroke volume, ml, during work in the supine position in relation to PWC_{170} in the supine position. Straight line represents the normal regression line. Dotted lines represent $\pm$ one standard error of estimate. The normal values are obtained from determination on 18 healthy subjects (HOLMGREN *et al.* 1960).

The cardiac output at rest amounted to 7.89 l/min (range 5.25—10.82) in the supine and 5.85 l/min (range 4.52—7.04) in the sitting position. On the highest work load, this increased up to 17.93 l/min (range 15.08—21.32) and

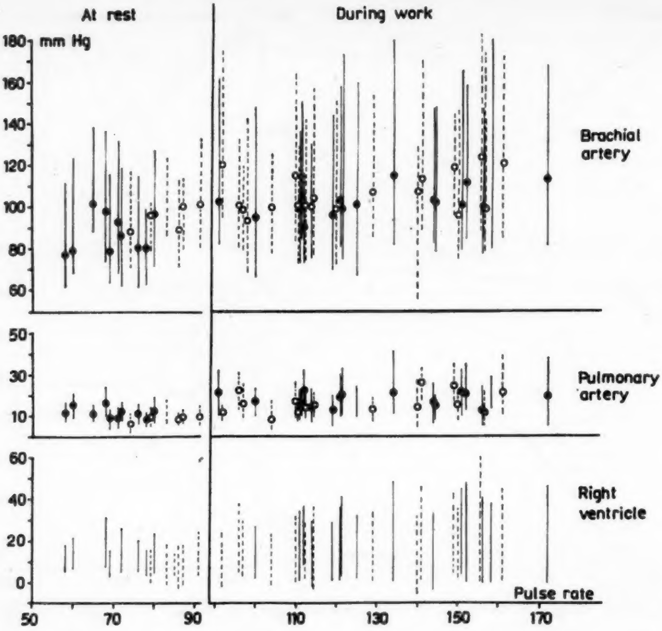

Fig. 11. Pressures, mm Hg, from brachial artery, pulmonary artery and right ventricle at rest during work. The lines represent systolic and diastolic pressures and the circles represent mean pressures. Straight lines and filled circles refer to values in the supine position and dotted lines and open circles to values in the sitting position.

15.94 l/min (range 13.31—17.80) respectively. The cardiac output at rest, as well as during work, was in each case lower in the sitting than in the supine position (Fig. 6 and 7). The differences were statistically significant and of approximately the same magnitude at rest and during the two work loads (Table II). The cardiac output, in the sitting posture at rest and during work, was on the average 2.22 l/min lower than in the supine posture (Fig. 9, Table II).

The stroke volume, at rest, was 115.7 ml (range 97—159) in the supine and 70.0 ml (range 57—81) in the sitting position. The greatest difference was noted in subject no. 3 where the stroke volume decreased 97 ml, corresponding to 55 per cent, on changing from a supine to a sitting posture. In the recumbent position, no significant differences in the stroke volume were found on changing from rest to work, nor during continued work with increasing intensity (Fig. 9).

In the sitting position, the stroke volume at rest was on the average 40 per cent lower than in the supine position. It increased considerably with mild work. With increased work it remained constant, but on a significantly lower

Table III. Pressures, mm Hg, at rest and during exercise

RV = Right ventricle. PA = Pulmonary artery. Br. A. = Brachial artery. S = Systolic. D = Diastolic. D_E = End diastolic. M = Mean. Re = Recumbent. Si = Sitting, $\overline{X}$ = Average. S.D. = Standard deviation. n = number of cases.

			Rest			Work load I			Work load II		
			$\overline{X}$	S.D.	n	$\overline{X}$	S.D.	n	$\overline{X}$	S.D.	n
RV	S	Re	22.8	5.7	8	32.0	5.7	8	44.4	7.7	8
		Si	18.6	3.1	7	30.3	5.4	7	41.9	10.0	7
	D_E	Re	5.1	1.6	8	1.5	3.2	8	0.3	2.2	8
		Si	0.2	2.4	6	0.4	3.0	7	0.3	3.1	7
PA	S	Re	19.2	4.5	10	27.8	5.9	9	35.8	8.1	9
		Si	13.8	2.2	6	21.3	5.5	8	26.8	7.6	8
	D	Re	7.3	2.2	10	9.3	2.8	9	9.4	3.8	9
		Si	5.2	1.7	6	8.6	2.6	8	10.3	5.3	8
	M	Re	12.4	2.7	10	16.4	3.5	9	18.3	3.5	8
		Si	8.7	1.6	6	14.1	4.3	8	17.5	5.5	8
Br.A.	S	Re	123.3	14.0	10	148.1	19.4	9	161.7	12.9	9
		Si	125.0	20.1	7	143.1	19.0	9	154.3	17.3	9
	D	Re	71.5	7.1	10	77.3	8.5	9	81.6	3.7	9
		Si	77.3	16.4	7	78.9	7.6	9	80.7	11.8	9
	M	Re	93.4	7.1	10	100.8	8.5	9	106.7	6.9	9
		Si	98.0	7.5	7	103.9	8.3	9	109.2	10.1	9

level than in the supine position (Fig. 8 and 9, Table II). The mean increase in the stroke volume on transition from rest to work in the sitting position amounted to 51 per cent. The stroke volume in the supine position amounted on the average to 13.2 per cent of the heart volume and 2.07 per cent of the blood volume, which is in agreement with values reported by HOLMGREN *et al.* 1960.

The intracardiac and intravascular pressures in these cases are presented in Fig. 11 and Table III.

It has not been possible to obtain exactly the same reference level in both positions. Therefore, the pressures in the sitting and the supine positions are not directly comparable. The brachial arterial pressure was within normal ranges both at rest and during work (HOLMGREN 1956). The pulmonary arterial wedge pressure was within normal limits in all subjects in which it was recorded. The pressures in the pulmonary artery and the right ventricle were also normal both at rest and during work when compared with a larger number of normal subjects (HOLMGREN *et al.* 1960). In the supine position,

Table IV. Data obtained during right heart catheterization in 10 healthy male subjects

S = sitting R = recumbent St. = standing Br. A = brachial artery RV = right ventricle D = diastolic M = mean

Case no. Cath. no.	Position	Work load kpm/min	Pulse rate beats/min	Oxygen uptake ml/min	Mechanical efficiency per cent	O_2-capacity ml/100 ml	O_2-sat. per cent Br. A.	O_2-sat. per cent P. A.	AV-O_2-diff. ml/L
1	R	rest	67	286	—	20.43	100	80	43.8
21/59	St	rest	100	344	—	20.61	97	75	36.9
	R	rest	60	311	—	21.17	96	80	38.8
	R	300	100	1,012	20	20.93	99	66	70.8
	R	600	125	1,540	23	21.05	99	56	94.1
	R	900	158	2,253	22	21.58	99	47	112.6
2	R	rest	69	315	—	18.48	98	79	39.4
26/59	St	rest	91	322	—	18.87	98	60	75.1
	R	rest	71	313	—	17.92	98	77	37.4
	S	300	98	1,054	19	18.60	100	50	91.4
	S	600	120	1,565	23	19.34	99	44	110.1
	S	900	156	2,196	23	19.75	98	38	119.5
3	R	rest	68	342	—	19.10	97	82	31.6
30/59	R	rest	67	352	—	19.49	96	79	33.7
	R	300	91	1,016	21	19.60	95	60	69.7
	R	900	134	2,119	24	20.19	94	45	99.4
	S	rest	86	383	—	20.34	97	68	62.2
	S	300	92	952	23	20.34	96	53	90.3
	S	900	129	2,007	26	20.69	97	38	123.0
4	R	rest	76	274	—	15.15	98	76	31.5
44/59	S	rest	91	323	—	16.04	97	66	54.6
	S	250	112	806	22	16.42	97	50	79.5
	S	500	150	1,264	24	16.39	96	40	95.0
	R	250	119	829	21	15.68	98	54	71.6
	R	500	156	1,306	23	15.68	95	41	86.6
5	R	rest	78	312	—	19.22	98	80	40.4
50/59	S	rest	87	381	—	19.52	98	71	54.1
	S	400	104	1,165	22	20.93	97	49	104.9
	S	800	140	2,007	22	20.69	98	40	119.9
	R	rest	75	318	—	18.90	99	77	43.7
	R	400	114	1,296	19	19.81	96	52	87.6
	R	800	144	2,116	21	19.96	98	42	112.1
6	R	rest	78	267	—	17.52	99	82	32.9
75/59	S	rest	83	313	—	18.17	99	72	50.3
	S	400	110	1,020	25	18.73	97	44	100.0
	S	800	161	1,847	24	19.31	99	34	127.9
	R	rest	80	242	—	17.35	101	84	29.9
	R	400	121	1,137	22	18.26	101	52	90.4
	R	800	172	1,897	23	18.84	97	39	109.7

ventricle

PA = pulmonary artery PCV = pulmonary capillary venous S = systolic De = end-diastolic

AV-O_2-diff. ml/L	Card. output L/min	Stroke volume ml	Pressures, mm Hg								
			RV		PA			PCV	Br. A		
			S	De	S	D	M	M	S	D	M
43.8	6.53	98	20	4	18	8	12	11	120	62	82
36.9	9.32	93	17	—	16	—	9		115	70	89
38.8	8.02	134	21	6	21	9	15		124	68	79
70.8	14.29	143	27	2	23	10	17		148	67	95
94.1	16.37	131	31	1	24	9	—		160	67	101
112.6	20.00	127	38	0	28	13	—		179	80	—
39.4	7.99	116			14	6	9	5	127	66	88
75.1	4.29	47			14	3	6		117	66	78
37.4	8.37	118			14	4	9		132	68	93
91.4	11.53	118							143	68	94
110.1	14.21	118							151	73	99
119.5	18.38	118							173	85	—
31.6	10.82	159	31	7	24	10	16	11	137	74	98
33.7	10.44	155	31	4	28	10	18	10	142	76	100
69.7	14.58	161	—	—	32	10	21	13	162	82	103
99.4	21.32	159	48	0	42	11	21	15	180	82	115
62.2	6.16	72	17	−3	11	6	8	—	113	71	89
90.3	10.54	114	24	−2	15	8	11	—	175	95	120
123.0	16.32	126	33	0	19	7	13	—	154	86	107
31.5	8.70	115	20	5	15	6	11	9	115	61	80
54.6	5.92	65	24	3	14	5	9		133	80	101
79.5	10.14	91	29	2	20	9	14		142	75	101
95.0	13.31	89	35	2	20	8	15		147	75	96
71.6	11.58	97	29	1	20	5	13		144	70	96
86.6	15.08	97	39	0	24	5	12		147	78	100
40.4	7.72	99	15	3	11	5	8	6	97	63	80
54.1	7.04	81	18	−1	13	6	9		114	80	100
104.9	11.11	107	24	−2	17	4	8		125	78	100
119.9	16.74	119	31	−6	27	4	14		128	56	107
43.7	7.28	97	—	—	—	—	—		—	—	—
87.6	14.79	130	29	−2	23	7	14		130	76	100
112.1	18.88	131	33	−4	26	6	16		147	83	103
32.9	8.12	104	24	7	19	8	14		129	72	99
50.3	6.22	75	18	−2	18	6	—		124	86	—
100.0	10.20	92	31	0	26	9	17		165	84	115
127.9	14.44	89	45	1	38	9	21		172	85	120
29.9	8.09	101	23	4	19	7	12		127	72	97
90.4	12.58	104	36	1	30	10	19		158	81	103
109.7	17.29	101	46	0	38	5	19		167	81	113

Table IV. cont.

Case no. Cath. no.	Position	Work load kpm/min	Pulse rate beats/min	Oxygen uptake ml/min	Mechanical efficiency per cent	O_2-capacity ml/100 ml	O_2-sat. per cent		AV-O_2-diff. ml/L
							Br. A.	P. A.	
7	R	rest	58	300	—	17.59	95	70	46.2
84/59	S	rest	85	389	—	18.15	96	57	74.3
	S	450	96	1,384	20	18.27	96	34	115.5
	S	900	141	2,200	23	18.78	95	30	123.6
	R	rest	64	293	—	17.10	94	70	40.7
	R	450	111	1,279	22	18.18	95	45	93.0
	R	900	152	2,109	24	18.99	95	34	115.8
8	R	rest	65	312	—	18.41	98	77	38.7
88/59	S	rest	74	390	—	18.93	97	63	66.4
	S	450	110	1,258	23	19.24	97	42	106.3
	S	900	156	2,172	23	19.32	95	30	123.2
	R	rest	58	316	—	17.60	97	75	40.0
	R	450	111	1,274	22	18.29	97	48	90.6
	R	900	144	2,040	25	18.92	95	36	110.7
9	R	rest	72	276	—	18.62	99	80	39.7
89/59	S	450	114	1,246	22	20.21	97	41	116.6
	S	900	156	2,050	24	20.05	98	31	135.5
	R	rest	62	276	—	18.32	99	79	38.6
	R	450	121	1,210	23	19.26	98	49	95.9
	R	900	165	2,026	24	19.79	99	36	123.9
10	R	rest	69	258	—	18.60	98	81	38.6
61/59	S	rest	79	254	—	19.31	98	71	56.2
	S	450	97	1,166	24	20.60	100	45	119.2
	S	900	149	2,122	23	21.18	98	37	131.5
	R	rest	72	241	—	19.05	98	77	45.9
	R	450	112	1,243	22	19.03	98	50	93.0
	R	900	151	2,069	24	20.00	98	41	115.6

the end diastolic pressure of the right ventricle decreased about 5 mm Hg during exercise. In the sitting posture, it was lower and remained constant during work.

Discussion

The methods used in this study involved procedures which could have changed the functions that were being examined. The introduction of the catheters, however, was made rapidly and without discomfort to the subject. The resting values for pulse rate and oxygen uptake were at levels usually

Card. output L/min	Stroke volume ml	Pressures, mm Hg								
		RV		PA			PCV	Br. A		
		S	De	S	D	M	M	S	D	M
6.49	112	18	5	16	8	11	5	112	62	77
5.24	61	13	3	—	—	—		—	—	—
11.98	125	38	5	30	13	22		132	80	101
17.80	126	46	1	33	18	26		169	89	113
7.20	113	24	3	18	7	12		114	64	84
13.75	124	34	1	23	8	16		137	74	99
18.21	120	48	0	35	13	20		158	85	111
8.05	124			15	8	11	9	139	88	110
5.87	79			10	2	6		117	71	88
11.83	108			16	7	11		129	73	101
17.63	113			21	5	11		141	78	99
7.90	136			18	9	13		133	82	102
14.07	127			23	10	16		151	85	105
18.43	128			19	8	15		148	78	102
6.95	97	26	5	16	8	13	9	119	62	86
10.69	94	36	−3	20	10	14		157	77	104
15.13	97	60	0	—	—	—		183	88	123
7.16	115	29	7	22	11	16		126	72	93
12.62	104	41	3	33	11	20		174	75	99
16.35	99	58	3	42	14	22		180	89	116
6.68	97	15	3	13	4	9	5	116	64	79
4.52	57	15	0	14	5	9		102	79	96
9.78	101	30	3	26	9	16		120	80	99
16.14	108	43	4	35	16	24		144	96	119
5.25	74	17	5	25	7	12		109	78	93
13.37	120	36	8	32	15	22		116	73	90
17.90	119	45	3	32	10	21		165	79	100

found in non-fasting and non-hospitalized individuals. The relationship between pulse rate and work load was the same both before and during heart catheterization (Table I). Therefore, the introduction of catheters does not seem to have changed the condition of the subjects. While performing exercise during heart catheterization, however, several subjects were not in a steady state with regard to the pulse rate on the highest load. One subject felt faint in the sitting position. The second work test started 30 min after the end of the first. At that time, restitution of pulse, oxygen uptake and cardiac output was obtained. In four subjects, the hemoglobin concentration was somewhat lower than the average normal value, but was not abnormally low. The per-

formance of these subjects did not deviate from that of the others with regard to the results of this study.

The influence of body position on the circulation at rest. With change of posture from supine to sitting, the arterio-venous oxygen difference increased and the cardiac output decreased (on an average of 2.0 l per min). At the same time, the pulse rate increased and therefore the stroke volume decreased considerably — on an average of 40 per cent. This is in agreement with earlier reports (McMichael and Sharpey-Schafer 1944, Stead *et al.* 1945, Lagerlöf *et al.* 1951, Donald *et al.* 1953). The decrease in stroke volume can be the result of an impaired diastolic filling of the ventricles or a less complete ejection.

In our subjects, the end diastolic pressure in the right ventricle was 5 mm Hg lower in the sitting than in the supine position. It is, however, uncertain if the reference level for zero pressure had the same relation to the right ventricle in the two body positions. Consequently, this difference between the filling pressures is doubtful.

It has been well established that with change from the supine to the sitting posture there is a shift of blood from the thorax to the legs and a decrease of the heart volume (Sjöstrand 1953, Weissler *et al.* 1959, Holmgren and Ovenfors 1960). An impaired diastolic filling, therefore, seems to explain the decrease in the stroke volume.

The influence of body position on the circulation during exercise. In the supine position, the arterio-venous oxygen difference and the cardiac output increased with increasing work intensities. This occurred to the same extent as reported in a larger series from this laboratory as well as by other investigators (see Holmgren *et al.* 1960). Generally the stroke volume did not vary during the change from rest to work and with increasing work loads. In the sitting position, the arterio-venous oxygen difference was higher and the cardiac output lower than in the supine position, even during work. The stroke volume increased considerably during the transition from rest to mild work. With further increase of the work load, the stroke volume increased only slightly. Even on the highest load, however, it was lower than in the supine position.

With the start of leg exercises in the sitting position, blood is redistributed from the leg veins to the thorax improving the diastolic filling of the ventricles. In our subjects, only the filling pressure of the right ventricle was determined and it was found to be unchanged during the transition from rest to exercise in the sitting position (Table III), cf. Holmgren (1956). The effective filling pressure was not measured, however, and the mean intrathoracic pressure might have changed during exercise. In the supine position, the absolute diastolic pressure in the right ventricle decreased during exercise. Probably this was caused by a decrease of the intrathoracic pressure, cf. Holmgren (1956). Therefore, a constant absolute filling pressure in the sitting position may be measured while the effective filling pressure increases when exercise is begun. It has been demonstrated earlier that the heart volume increases

during the transition from rest to work in the sitting position (Holmgren and Ovenfors 1960) which is a sign of improved diastolic filling of the ventricles.

The relationship between the stroke volume and PWC_{170}

Holmgren *et al.* (1960) have demonstrated a good correlation between stroke volume and dimensional parameters of the circulatory system such as heart volume, total amount of hemoglobin and PWC_{170}. To diminish the random variation of the stroke volume due to biological variation (Holmgren and Pernow 1960), a mean value for the stroke volume during exercise in the supine position was calculated from the observations available. In our cases, the value for the stroke volume obtained in this manner was plotted against the PWC_{170} in the supine position and showed close correlation (Fig. 10).

Even during the highest work load the stroke volume was somewhat smaller in the sitting position than in the supine. But the work performed at a pulse rate of 170 beats per minute was the same in the supine and sitting positions. This is explained by a better utilization of the oxygen in the sitting position, giving an oxygen pulse (oxygen transport per heart beat) of the same size as in the supine position.

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